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Chemical genetic manipulation of interferon regulatory factor 1 (IRF-1) using synthetic biology

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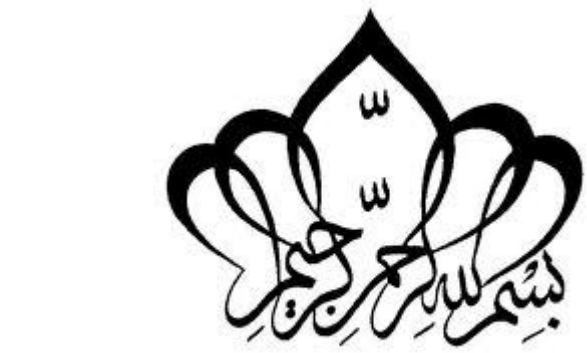
Doctor of Philosophy



My Lord, I ask you to enhance my knowledge

Taha [20:114]

To my sweet wife

To my lovely angels

I. Declaration

I confirm that I have composed this thesis. The majority of the work is my own and contributions by other members of my research group have been clearly indicated. This work has not been submitted for any degree of professional qualification other than this PhD.

Khaldoon M. Alsamman

Signature:

Date:

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II. Acknowledgment

A massive thank you for my PhD supervisors: Prof. Ted Hupp, Prof. Kathryn Ball, and Dr. Peter Hohenstein. Thank you Ted for giving me this opportunity to do my PhD in your lab, and thank you for being very optimistic and positive about my results. Thank you Kathryn for your great and lovely ideas on work related to IRF-1; without your input, this wasn't possible. Thank you Peter for teaching me all recombineering tricks and introducing me to the world of genetics.

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III. Abstract

Interferon regulatory factor 1 (IRF-1), the founding member of IRF family, is a nuclear transcription factor first described as a transcription factor that binds to the upstream region of interferon induced genes following viral infection. In addition, IRF-1 has been reported to be involved in cell growth regulation, induction of apoptosis, immune responses, post-transcriptional modification, and cell transformation by oncogenes. Thus, IRF-1 shows accumulative evidence supporting the theory that IRF-1 functions as a tumour suppressor.

However, we still lack the knowledge in the regulation and function behind IRF-1 and many other tumour suppressors due to the lack of synthetic tools that can aid in understanding the mechanism of cancer biology. Here we described the creation of synthetic tools that can be applied to study the role of a transcription factor(s) in cancer biology.

Firstly, we described the creation, using recombineering technology, of universal bacterial artificial chromosome (BAC) targeting vector. This targeting vector, carry a *cre*-conditioned STOP cassette that can be targeted at a desired specific area. The resulted targeting vector can aid the generation of mice models with a conditioned knock-in subtle mutation(s). The resulted *cre*-conditioned mice models are an essential tool for any outstanding research project in cancer biology.

Secondly, we described the development of Flp-In System™ from Invitrogen; the system can ease the generation of isogenic stable mammalian expression cell lines. Using this system, we created two isogenic stable cell lines expressing wild-type IRF-1 and a mutant that abolish IRF-1 DNA binding ability (W11R). Both cell lines were investigated using microarray analysis revealing new IRF-1 target genes.

We reported the up-regulation of expected standard interferon regulatory genes such as, interleukin-24 (*IL-24*) and interferon regulatory factor-2 binding protein-2 (*IRF2BP2*) and the up-regulation of standard apoptotic genes such as, early growth response-1 (*EGR-1*) and prostate transmembrane protein, androgen induced-1 (*PMEPA1*) confirming the role of IRF-1 as a tumour suppressor. However, we also reported the up-regulation of secreted phosphoprotein-1 (*SPP1*) and SH3 and PX domains-2A (*SH3PXD2A*) which are matricellular protein produced by cancer cells playing a role in cellular adhesion, invasion, tumour growth progression and metastasis. Thus, we proposed a new biological role of IRF-1 in cellular movement.

Thirdly, we described the development of a synthetic stable reporter cell line which can report IRF-1 transcriptional activity; such reporter cell line can be used once large scale screening is needed. The created stable reporter cell line was used to screen a kinase inhibitor library which has revealed C3 as an IRF-1 modifier. The newly identified IRF-1 modifier regulates IRF-1 transcriptional activity by inhibiting platelet-derived growth factor receptor (PDGFR) and/or vascular endothelial growth factor receptor (VEGFR) tyrosine kinase.

Finally, we validated the synthetic Flp-In System™ by testing the system using a novel oncoprotein model. We have developed a stable cell line that overexpresses an oncoprotein named Anterior Gradient 2 (AGR-2). We have found that AGR-2 can attenuate IRF-1 protein levels dependent of p53. In addition, AGR-2 has been identified as a cellular survivor factor during unfolding protein response.

In conclusion, this study descried the creation and the validation of synthetic tools: synthetic cassette for *cre*-conditioned mice creation, the Flp-In System™ for isogenic stable cell line creation, and IRF-1 reporter cell line for high throughput screening. All synthetic tools were validated and used to investigate IRF-1, a transcription factor that plays a role in cancer and immune system.

IV. Abbreviation

AGR-2	Anterior gradient 2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
BAC	Bacterial artificial chromosome
BP	Gateway® BP clonase® enzyme mix
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CHIP	C terminus of Hsc70-interacting protein
CHOP	CCAAT/enhancer binding protein (C/EBP), epsilon
CK2	Casein kinase 2
cRNA	Complementary ribonucleic acid
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FLT3	Fms-related tyrosine kinase 3
FRT	Flp recombination target
GAF/AAF	Interferon-γ activated factor/interferon-α activated factor
GC	Glucocorticoids
GRIP-1	Glutamate receptor-interacting protein 1
Hsp	Heat shock protein
HTS	High throughput screening
IC_{50}	The half maximal inhibitory concentration
ICSBP	Interferon consensus sequence binding protein
IL	Interleukin
INF-α	Interferon-alpha
INF-β	Interferon-beta
INF-γ	Interferon-gamma
iNOS	Nitric oxide synthase
IRF	Interferon regulatory factor
IRF-E	Interferon regulatory factor element
ISG-20	Interferon stimulated gene 20
ISGF3	Interferon stimulated gene factor 3
ISGs	Interferon stimulated genes
ISRE	Interferon sensitive response element

J/m^2	Joule per meter squared
Jak1	Janus kinase 1
kDa	Kilodalton
KEGG	Kyoto encyclopedia of genes and genomes
KI	Knock-in
KO	Knock-out
KPNA2	Karyopherin alpha 2
LB	Luria-Bertani broth
LR	Gateway® LR clonase® enzyme mix
MAPK	Mitogen-activated protein kinase
MDA-7	Melanoma differentiation-associated gene-7
MEFs	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary gene 88
NK	Natural killer
nm	Nanometer
NPM	Nucleophosmin
ORF	Open reading frame
PAC	P1 artificial chromosome
PBS	Phosphate buffer saline
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEL	Primary effusion lymphoma
PKA	Protein kinase A
PKR	Protein kinase R (RNA activated)
PPS	Popliteal pterygium syndrome
PRDI	Positive regulatory domain I
QC	Quality check
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated on activation normal T-cell expressed and secreted
rpm	Round per minute
RSN	Robust spline normalisation
RT	Reverse transcription
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
RVD	Repetitive variable di-residues
S33Y	Serine 33 to tyrosine
S45Δ	Serine 45 deletion
SDS	Sodium dodecyl sulfate
SLE	systemic lupus erythematosus
SPP1	Secreted phosphoprotein 1
ssDNA	Single-stranded deoxyribonucleic acid
SUMO	Small ubiquitin-like modifier

TALENs	Transcription activator-like effector nucleases
TALEs	Transcription activator-like effectors
TAT	Trans-activator of transcription
TLR3	Toll-like receptor 3
TPP	Tecno plastic products
Tyk2	Tyrosine kinase 2
UPS	Ubiquitin-proteasome system
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VRE	Virus response element
VST	Variance stabilizing transformation
W11R	Tryptophan 11 to arginine
YAC	Yeast artificial chromosome
ZFNs	Zinc-finger nucleases

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Chapter 1: Literature review

1.1 Introduction

According to the World Health Organization, cancer is the second most common cause of death in the developed world; only cardiovascular disease mortality exceeds that of malignancy. The last two decades have seen enormous advances in our understanding of the cellular and molecular basis of malignancy, its initiation, promotion, and progression. There is no single molecular cause of cancer, and for each different cancer type, many genes are involved.

Scientists and clinicians have become more interested in cancer biology due to recent developments in molecular biology, genetics, virology and immunology leading to new and better approaches for studying the nature of cancer cells and the mechanism of tumour induction. Here we will review the basic pathology of cancer, followed by a brief introduction on the interferon system and interferon regulatory factors. Finally, a detailed description of a distinct tumour suppressor gene termed interferon regulatory factor 1 (IRF-1) and its cooperation in tumorigenesis will be highlighted.

Cancer is a broad medical term that describes a group of diseases that involves unregulated cellular growth. Cancer is a cellular state in which cells grow and divide uncontrollably forming a tumour which could be malignant or benign. Malignant tumours can invade nearby parts of the body and spread to more distant locations through the lymphatic system and/or bloodstream. Cancer Research UK indicated there are more than 200 types of cancer which can develop in more than 60 organs.

Many factors can increase the risk and contribute toward cancer, including internal factors, such as inherited mutation, hormones, and immune condition, and environmental factors, such as smoke exposure, alcohol consumption, diet, radiation, and infectious organism (Anand *et al*., 2008). All cancers result from multiple mutations (Loeb and Loeb 2003) where these factors directly or indirectly can cause a defect in an oncogene and a tumour suppressor gene resulting in uncontrolled cellular growth where cells keep growing and multiplying without regulation.

1.2 The interferon system

Interferons (INFs) were first reported by Isaacs and Lindenmann back in 1957 as proteins that could make other cells resistant to viruses following infection of cells with a supernatant from other cells already infected with the same virus (Isaacs and Lindenmann 1957). Today, interferons are known to be a class of proteins produced by cells in response to antigen such as viral infection or any external agent, and act as a natural barrier in order to protect the body from infections. Interferons are considered to be cytokines belonging to the group of interleukins and they are classified into four types: interferon-α, interferon-β, interferon-γ and recently interferon-λ. Both interferons α and β are categorized into subclass I, whereas interferon-γ is considered to be in subclass II and finally interferon-λ is classified as subclass III. Interferons are well studied for their role in antiviral response, immune modulation, haematopoietic development and their antiproliferative response by regulation of the cell cycle (Taniguchi *et al*., 2001; Nguyen *et al*., 1997; Stark *et al*., 1998).

1.2.1 The old interferons

Till a few years ago, interferons were thought to be divided into the type I and type II subgroups only. Interferon-α or "leukocyte" interferon are cytokines to type I interferon having multiple effects on cell function (Vilcek, 2006). The interferon-α family is composed of at least 13 interferon subtypes, coded by more than 15 genes and 9 pseudogenes found on human chromosome 9 and murine chromosome 4 (De Andrea *et al.*, 2002). Interferon-α genes do not contain introns and the whole gene codes for the interferon-α protein (De Andrea *et al*., 2002). All interferon-α proteins are produced from macrophages, particularly lymphocytes, cells that use similar receptor systems and have similar biological activities. Interferon-α has been used in clinical oncology for treating more than 14 different cancer types (Ferrantini *et al*., 2007) including haematological malignancies and certain solid tumours such as melanoma and carcinoma (Ferrantini *et al*., 2007).

Despite many years of interferon-α research in animal models, the different mechanism of action is still a matter of debate. The direct inhibitory effect on tumour cell growth and function was the major anti-tumour response seen as a result of treating patients with interferon-α. In fact, interferon-α can directly down-regulate oncogene expression, induce tumour suppressor activity, and inhibit the proliferation of normal and tumour cells *in vitro* and *in vivo* (Ferrantini *et al*., 2007).

Interferon-β or "fibroblast" interferons are cytokines of the type I interferon family with interferon-α. Interferon-β is mainly produced in epithelial cells and fibroblasts under the action of foreign nucleic acids, such as viral genomes or other types. Interferon-β is also coded by a gene with no introns found on human chromosome 9 (De Andrea *et al*., 2002). However, interferon-γ belongs to a separate subclass, so-called type II interferon, named as "immune" interferon. Interferon-γ is produced especially by lymphocytes sensitized with the help of macrophages and only under the action of external mitogens. Unlike interferon-β, interferon-γ is transcribed from a single gene containing three introns located on human chromosome 12 (De Andrea *et al*., 2002).

1.2.2 The new interferon

Interferon-λ was just identified few years ago and classified as a new group: type III interferon. In humans, there are three distinct interferon-λ proteins termed INF-λ1, INF-λ2, and INF-λ3 (Donnelly and Kotenko 2010). They are also named interleukin-29 (IL-29), IL-28A, and IL-28B, respectively (Sheppard *et al.*, 2002). The members of this new interferon family were found to interact through unique receptors (distinct from those used by type I and type II interferons) composed of the unique IFN-λR1 chain, called IL-28AR, and the IL-10R2 chain which is shared with IL-10, IL-22, and IL-26 receptor complexes (Lasfar *et al*., 2011).

Although type III interferons bind to a specific receptor, the downstream signalling is similar to that induced by type I interferon and consists of the activation of janus kinase (Jak1) and tyrosine kinase 2 (Tyk2) kinases (section 5.2.1) leading to the activation of the interferon stimulated gene factor 3 (ISGF-3) transcription complex (Lasfar *et al*., 2011).

Although type I and type III interferons induce similar cell signalling, the intensity of cell signalling as measured by signal transduction and transcription (STAT1) activation appeared to be lower for type III interferon (Lasfar *et al*., 2006). In addition, antiviral studies *in vivo* and *in vitro* have shown that both interferon-α and interferon-λ contribute toward antiviral response (Ank *et al*., 2006), and other studies showed that type III interferon can inhibit the replication of hepatitis C and B viruses (Hong *et al*., 2007), which suggests an alternative to interferon-α treatment among patients with interferon-α resistance hepatitis. Another group showed that interferon-λ can inhibit human immunodeficiency virus type 1 (HIV-1) infection of blood monocyte-derived macrophages (Hou *et al*., 2009) suggesting that interferon-λ exerts immunomodulatory effects that overlap those of type I interferons (Lasfar *et al*., 2011).

In addition to antiviral and immunomodulatory activity, type I interferons demonstrate antiproliferative activities in most cell lines, whereas this activity is restricted within interferon-λ (Li *et al*., 2009). Type I interferons have been shown to induce apoptosis in cancerous cells by inducing a quick and potent signalling leading to the expression of more than 300 interferon stimulated genes (ISGs) (Der *et al*., 1998), many of which are involved in encoding apoptosis related proteins (Clemens 2003). However, interferon-λ does not inhibit the proliferation of many types of tumour cell lines, though some reports showed an inhibition in some cell lines, such as human glioblastoma (Meager *et al*., 2005).

1.3 The interferon regulatory factors

A key factor in interferon induce response is the interferon regulatory factors (IRFs). IRFs are a group of transcription mediators of bacteria, viruses, and interferon-induced signalling that result in different responses within immune response, cell growth regulation, and apoptosis. To date, nine members belong to the human IRF family including IRF-1, IRF-2, IRF-3, IRF-4 (Pip or ICSAT), IRF-5, IRF-6, IRF-7, IRF-8 (ICSBP), and IRF-9 (ISGF3γ or p48). In addition, IRF-10 was identified in chicken; however, it is absent in humans and mice (Nehyba *et al*., 2002). All ten factors share significant homology in the N-terminal 115 amino acids, which contain the DNA binding domain characterized by five tryptophan repeats (Figure 1.1).

The C-terminal regions of most IRFs carry an IRF association domain (IAD) that is responsible for homo- and heteromeric interaction with other family members or other transcription factors such as PU.1 and STAT (Taniguchi *et al*., 2001). However, the unique function of a particular IRF is influenced by a combination of cell-type specific expression and its ability to interact with other family members and transcriptional activators (Taniguchi *et al*., 2001).

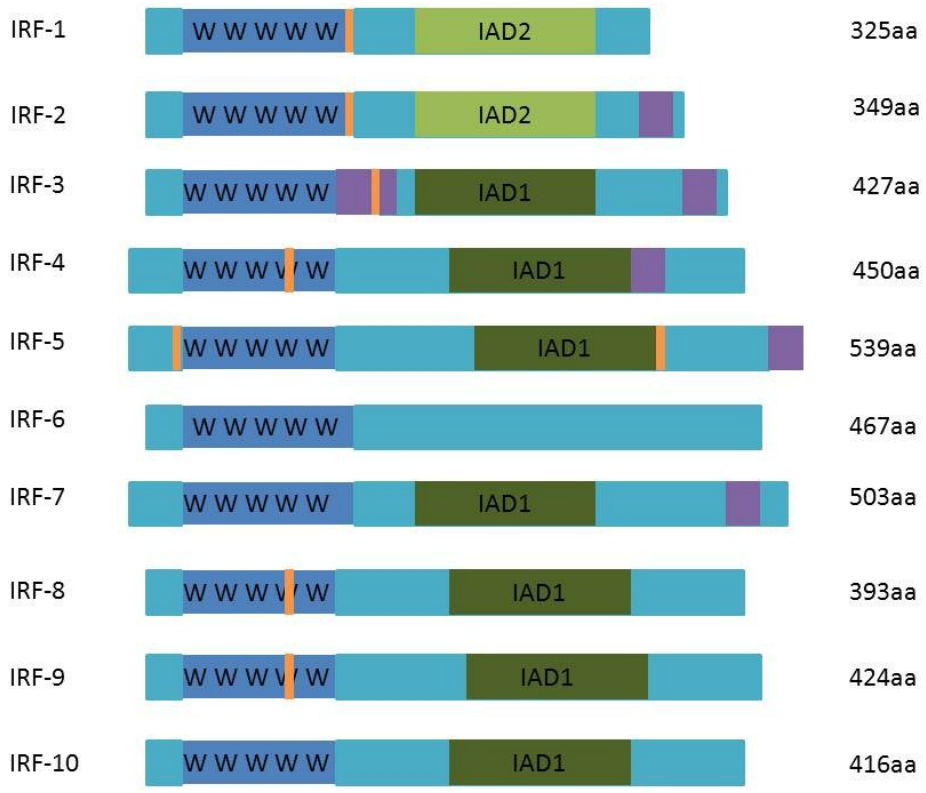


Figure 1.1: All interferon regulatory factors are composed of a DNA binding domain (navy blue) and a regulatory domain (light blue). For all IRFs, the DNA binding domain is characterized by 5 tryptophan repeats that are 10–18 amino acids apart. Most IRFs contain an IRF association domain (IAD) of either type I (Dark green) or type 2 (light green). Some IRFs contain a repression domain (purple) and a nuclear localization signal (orange). The size of each IRF in number of amino acids is indicated on the right.

1.3.1 The first IRF: IRF-1 and IRF-2

The first two members, IRF-1 and IRF-2, were discovered through their ability to bind to the positive regulatory domain I (PRDI) in the virus response element (VRE) of interferon-β genes, suggesting a function as an activator and repressor of interferon-β genes (Fujita *et al*., 1989); however, IRF-1 null mice did not have impaired activation of interferon-α and interferon-β genes in mouse embryonic fibroblasts (MEFs) (Reis *et al*., 1994). Further studies showed that IRF-1 is involved in antiviral defence mediated by interferon-γ, as the induction of nitric oxide synthase (iNOS) and guanylate binding protein was impaired in interferon-γ treated IRF-1 deficient MEFs (Kimura *et al*., 1994). Later, it was shown that IRF-1 is induced by interferon-γ and the induction of iNOS genes by interferon-γ is mediated by IRF-1 (Coccia *et al*., 2000). Further studies suggested that IRF-1 has an essential function in the development and activation of various immune cells (Duncan *et al*., 1996). In addition, IRF-1 also plays a critical role in the inducible expression of major histocompatibility complex (MHC) class I and apoptosis, as cells from IRF-1 null mice are resistance to UV and drug-induced apoptosis (Reis *et al*., 1994). In humans, polymorphism in IRF-1 has been associated with predisposition to asthma in the paediatric population (Wang *et al*., 2006), and deletions in IRF-1 has been observed in myelodysplastic syndrome and leukaemia (Willman *et al*., 1993).

Harada *et al* (1989) reported the discovery (by cross hybridization with IRF-1 cDNA) of another distinct interferon regulatory factor, IRF-2, that binds to the same *cis*-regulatory element of IRF-1 (Harada *et al*., 1989). IRF-1 and IRF-2 show 62% homology in the first 154 residues (amino-terminal region) and 25% homology in the remaining structure (Harada *et al*., 1989). IRF-2 overexpression in fibroblasts results in oncogenic transformation of these cells (Paun and Pitha 2007); also, IRF-1 has been shown to activate the transcription of histone 4 gene (Vaughan *et al*., 1995).

The role of IRF-2 in the innate antiviral defence has not been clearly established, results from IRF-2 null mice showed impaired natural killer (NK) cells, which displayed an immature phenotype and compromised receptor expression, suggesting that IRF-2 deficiency results in a defect in the late stages of NK cells maturation (Taki *et al*., 2005).

1.3.2 The antiviral IRF: IRF-3 and IRF-7

IRF-3 and IRF-7 are closely related to each other based on their primary structure (Figure 1.1). IRF-3 was discovered by searching the expressed sequence tag database for IRF-1 and IRF-2 homologues (Au *et al*., 1995). IRF-3 mRNA levels were found to be expressed constitutively in all tissues and not induced by viral infection or interferon treatment (Au *et al*., 1995). IRF-7 discovery was reported by Zhang *et al* (1997), and its expression is considered ubiquitous; however, it's totally dependent on type I interferon signalling, unlike IRF-3 (Marie *et al*., 1998 and Sato *et al*., 1998). The role of their transcriptional activation of interferon genes and the identification of IRF-3 and IRF-7 had an outbreak in understanding the mechanism in which a pathogen induces innate antiviral response (Ronco *et al*., 1998; Au *et al*., 1998; Marie *et al*., 1998).

Upon recognition of dsRNA by a cellular receptor, IRF-3 is ubiquitously expressed (Ronco *et al*., 1998). The signalling pathway of Toll-like receptor 3 (TLR3) or the cytoplasmic RNA helicases (RIG-1) both characterized by the presence of caspase recruitment domain (CARD) will phosphorylate IRF-3 at the C-terminal region, at serine 386, using two non-canonical I_kB kinases, TBK-1 and IKKε (Fitzgerald *et al*., 2003). Further crystal structure showed IRF-3 activation by structural changes following that specific phosphorylation (Takahasi *et al*., 2003).

This activated IRF-3 will then homo/hetero dimerize with IRF-7 and translocate to the cellular nucleus where it associates with the CREB binding protein CBP and p300 to stimulate transcription of interferon-β genes and some other interferon stimulated genes such as *ISG-54* and *RANTES* (regulated on activation normal T-cell expressed and secreted) (Yoneyama *et al*., 1998 and Lin *et al*., 1999). Phosphorylated IRF-3 is under negative regulation by the ubiquitin proteasome pathway (Lin *et al*., 1998).

It has been shown that propyl isomerase (Pin1) targets activated IRF-3 for ubiquitin-mediated degradation (Saitoh *et al*., 2006). ISG-15, an interferon-induced ubiquitin-like protein, initiates the ubiquitin-mediated degradation of IRF-3, stabilizes IRF-3, and increases nuclear retention (Lu *et al*., 2006). Thus, ISG-15 plays an important role in enhancing host antiviral response.

IRF-7 was discovered as a factor binding to the Qp promoter of the Epstein-Barr Virus (EBV), and a splice variant of IRF-7 was identified as a key player in the induction of interferon-α genes (Zhang *et al*., 1997). Human IRF-7 is located on chromosome 11 at position 15.5 in a CpG-rich region that is methylated in some cancers (Lu *et al*., 2002). IRF-7 transcription can be induced using type I interferon and tumour necrosis factor (TNF-α) (Lu *et al*., 2002). Further results suggested that IRF-7 is a master regulator of type I interferon expression as IRF-7 overexpression in infected human fibroblasts, which express only interferon-β, conferred expression of several interferon-α genes (Yeow *et al*., 2000). In addition, mice with homozygous deletion of *IRF-7* were unable to express type I interferon genes following viral infection or activation of TLR9 using CpG-rich DNA (Honda *et al*., 2005).

IRF-7 is also phosphorylated by the signalling pathway mediated by TLR3, TLR7, TLR8 and TLR9, in which serine 477 and serine 479 appear to be critical targets for activation using TBK-1 kinase activity (Sharma *et al*., 2004); however, TLR7- and TLR9-stimulated phosphorylation of IRF-7 is dependent not on TBK-1 but rather on MyD88, IRAK-4, IRAK-1, and TRAF6 (Uematsu *et al*., 2005).

1.3.3 The hematopoetic IRF: IRF-4 and IRF-8

IRF-4 and IRF-8 show a degree of structural homology. Both are expressed mainly in lymphocytes, B-cells, and dendritic cells (Eisenbeis *et al*., 1995). Both proteins show a weak DNA binding affinity which can be increased with the association of other transcription factors (Tailor *et al*., 2006). IRF-4 has been characterized in the literature with different terminology: (i) Pip, a stabilized binding protein which heterodimerizes with the transcription factor PU.1, and where such heterodimerization was shown to bind to the IgG enhancer and activate the expression of the immunoglobulin (Ig) light chain in B-cells (Eisenbeis *et al*., 1995); (ii) LSIRF, a new IRF family member expressed in lymphoid cells (Mittrucker *et al*., 1997); and (iii) ICSAT, a factor that binds to the regulatory element of the human interleukin-5 gene (Yamagata *et al*., 1996).

IRF-4 has also been reported to be a natural antagonist of both IRF-1 and IRF-5 transactivation (Yoshida *et al*., 2005). Mice IRF-4 null models were deficient in mature T and B-cells suggesting a role of IRF-4 in such cells maturation (Lu *et al*., 2003). In addition, *IRF-4* null mice also showed a developmental block at several steps of T and B-cells differentiation (Mittrucker *et al*., 1997). The function of IRF-4 can also be revoked by an inability to associate with PU.1 transcription factor as observed in primary effusion lymphoma (PEL), which is characterized by an arrest in B-cell differentiation (Arguello *et al*., 2003), suggesting any defect in IRF-4 expression or function will lead to immune deficiency. In multiple myeloma cells, IRF-4 has been found to be translocated near the immunoglobulin heavy chain locus and consequently overexpressed, leading to the suggestion that uncontrolled expression of IRF-4 may lead to multiple myeloma phenotype (Iida *et al*., 1997).

IRF-8 or interferon consensus sequence binding protein (ICSBP) shares some similarities with IRF-4. The expression of IRF-8 is restricted to myeloid and lymphoid cell lineages and is induced by interferon-γ but not interferon-α or interferon-β (Driggers *et al*., 1990). IRF-8 binds DNA after interaction with other IRF members such as IRF-1 and IRF-2 and other transcription factors such as PU.1 and E47 (Politis *et al*., 1992).

However, the IRF-8 and IRF-1 transcriptional complex serves as a suppressor of transcription, whereas the IRF-8 and IRF-4 heterodimer activates transcription of ISG-15 (Meraro *et al*., 2002). The IRF-8 and IRF-1 complex also induces the transcription of genes for macrophage differentiation and macrophage-induced inflammation (Liu *et al*., 2004).

1.3.4 The surprising IRF: IRF-5 and IRF-6

Both IRF-5 and IRF-6 show completely different functions even though they share a similar structure. IRF-5 is induced by interferon-α and interferon-β stimulation (Tadatsugu *et al*., 2001) and has a role in apoptosis and the immune response to pathogen (Paun and Pitha 2007). However, IRF-6 has been reported to be a key player in the switch between keratinocyte proliferation and differentiation (Richardson *et al*., 2006). In addition, the IRF-6 null mouse model showed embryonic lethality with abnormal external morphology (Ingraham *et al*., 2006).

Human IRF-6 is located on chromosome 1 position q32, which is defined as the Van der Woude syndrome locus. This genetic disorder is associated with a nonsense mutation in *IRF-6* causing an autosomal dominant form of cleft lip and palate (Mostowska *et al*., 2005). Further mutation of *IRF-6* is also associated with popliteal pterygium syndrome (PPS) characterized by similar orofacial phenotype skin lesions and genital abnormalities (Kondo *et al*., 2002). These unexpected properties of IRF-6 indicate that IRFs may also have basic roles unrelated to the immune response and the immune system.

IRF-5 has been implicated in the innate inflammatory response. Human IRF-5 protein shows some properties distinct from IRF-3 and IRF-7: the IRF-5 protein structure contains two nuclear localization signals (Figure 1.1) and consequently nuclear IRF-5 can be detected in untransfected cells (Barnes *et al*., 2001). IRF-5 can be induced by type I interferon and viral stimulation (Mancl *et al*., 2005); also IRF-5, can be induced by the tumour suppressor p53 (Mori *et al*., 2002), suggesting a connection between these two proteins inducing pro-apoptotic pathways. Similar to p53, IRF-5 stimulates the cyclin-dependent kinase inhibitor $p21^{(WAF1/\ CIP1)}$, while repressing cyclin B1 and stimulating the expression of pro-apoptotic genes such as *Bak1*, *Bax*, *Caspase 8*, and

DAP kinase 2 (Hu *et al*., 2006). In addition, IRF-5 overexpression specifically up-regulates many early inflammatory genes including *RANTES*, *MIP-1β*, *I-309*, *MCP-1* and *IL-8*, suggesting a very important role for IRF-5 in early inflammatory cytokines and chemokines response, maturation, development, and expression. (Barnes *et al*., 2004).

Human IRF-5 protein is expressed in multiple splice variants, some of which are transcriptionally inactive and others function as dominant negative mutants (Mancl *et al*., 2005). Mutations in human IRF-5 have been associated with autoimmune diseases, such as systemic lupus erythematosus (SLE) which is characterized by interferon-α overproduction and polymorphism in the *Tyk2* and *IRF-5* genes. This observation suggests a connection between IRF-5 expression, interferon-α production, and autoimmunity (Graham *et al*., 2006).

1.3.5 The last IRF: IRF-9 and viral IRF

IRF-9, known in the literature as p48 or ISGF3-γ, plays an important role in the antiviral effect of type I interferon. IRF-9 forms a tertiary complex with interferon stimulated gene factor 3 (ISGF3), which binds to the interferon stimulated response element (ISRE) found in the regulatory element of interferon stimulated genes (Veals *et al*., 1992). Within this complex, STAT1 and STAT2 are included where IRF-9 is the major DNA binding component. In addition, IRF-9 can form further complexes by binding individually to STAT1 or STAT2 with the same DNA specificity as ISGF3 (Kraus *et al*., 2003). As IRF-1 and IRF-2, IRF-9 is expressed in a variety of tissues and shown to be essential for the antiviral response by interferon-α, interferon-β, and interferon-γ (Bluyssen *et al*., 1996).

Kaposi's sarcoma-associated herpes virus (KSHV) or human herpes virus-8 (HHV-8) is a member of the (γ) herpes virus family, and causes Kaposi's sarcoma and primary effusion lymphoma. Sequence analysis of the KSHV genome revealed the presence of about 80 different open reading frames (ORF) where few showed similarity to other genes that regulate cell growth, immune function, inflammation and apoptosis (Moore *et al*., 1998), including a cluster of four ORFs that showed homology to cellular transcription factor of the IRF family (Cunningham *et al*., 2003).

Three viral IRFs (vIRF) have been cloned and characterized. The first is the K9- vIRF-1 which encodes a hypothetical 449aa protein with 13.4% identity to human IRF-8 (Moore *et al*., 1998). vIRF-1 binds with several IRFs including IRF-1 and inhibits IRF-mediated transcriptional activation (Gao *et al*., 1997). vIRF-1 has been shown to inhibit both the virus-mediated induction of type I interferon genes and interferon stimulated genes, where its overexpression in fibroblasts initiates tumorigenicity when injected into nude mice (Gao *et al*., 1997). vIRF-1 binds to CBP/p300 and inhibits its acetyl transferase activity resulting in the global inhibition of histones H3 and H4 acetylation (Li *et al*., 2000). The vIRF-1 anti-apoptotic effect of vIRF-1 is thought to be related to p53, and IRF-1 (Burysek *et al*., 1999 and Seo *et al*., 2001).

The second vIRF is vIRF-2, which is encoded by ORF K11.1, makes a small nuclear protein with 163aa (Burysek *et al*., 1999). Unlike cellular IRF members, vIRF-2 binds to oligodeoxynucleotides corresponding to the NF-κB site and associates with other IRF members and p300 (Burysek *et al*., 1999). Also, vIRF-2 has been reported to bind to protein kinase RNA-activated (PKR) inhibiting its kinase activity and blocking the phosphorylation of its substrate, eukaryotic translation initiation factor 2α (Burysek *et al*., 2001).

The third vIRF characterized is vIRF-3 or LANA2 encoded by ORFs K10.5 and K10.6 (Lubyova and Pitha 2000). vIRF-3 is a multifunctional nuclear protein, and binds to IRF-3, IRF-7, and CBP/p300. In addition, vIRF-3 can also stimulate IRF-3/IRF-7 mediated transcription of type I interferon genes (Lubyova *et al*., 2000). Further, interaction of vIRF-3 with p53 results in inhibition of p53 mediated transcriptional activation and p53 induced apoptosis (Rivas *et al*., 2001).

1.4 Interferon regulatory factor 1

1.4.1 IRF-1 and cancer association

Ectopic IRF-1 overexpression in a wide range of different cell types obtained from many different mammalian species, such as human, mouse and hamster, has been reported to cause growth inhibition (Kirchhoff *et al*., 1993; Tanaka *et al*., 1996; Kirchhoff *et al*., 1996; Coccia *et al*., 1999). It was concluded that this suggested antiproliferative effect of IRF-1 was caused by the potential ability of IRF-1 to stimulate transcription of antiproliferative acting genes (Nguyen *et al*., 1997). IRF-1 causes cell growth inhibition by inducing interferon-β gene expression or other secreted factors (Kroger *et al*., 2002). Several IRF-1 induced antiproliferative genes have been characterized (Kroger *et al*., 2002), of which PKR is the most studied. PKR function is thought to prevent viral mRNA translation in infected cells. In addition to PKR, 2',5'-oligoadenylate synthetase (2',5'-OAS), whose product activates the mRNA degradation enzyme RNase L, is also induced by IRF-1 stimulation (Kroger *et al*., 2002). The products of many other IRF-1 induces antiproliferative genes lead to G_1 cell cycle specific arrest such as the tumour suppressor protein p53 and the cell cycle-dependent kinase inhibitor p21 (Kroger *et al*., 2002). The expression of many other genes, such as *IL-4*, *IL-5*, *IL-7* receptor, *E-cadherin*, *ISG*, and *MHC HIL* class I genes are induced by IRF-1 (Tanaka *et al*., 1993) and contributes to the growth-suppression effect. Thus, IRF-1 is considered to be a novel tumour suppressor.

Clinically, mutation in *IRF-1* genes has been observed in variety of human cancers. One of the most frequent cytogenetic abnormalities in human leukaemia and myelodysplasia (MDS) is an interstitial deletion within chromosome 5 location 31 (5q31) (Willman *et al*., 1993). This deletion has been reported to be the smallest commonly deleted site where *IRF-1* has been mapped (Willman *et al*., 1993).

Only *IRF-1* has been consistently deleted at one or both alleles in 13 cases of leukaemia or myelodysplasia within the area of 5q31. Thus *IRF-1* may be a critical deleted gene in human leukaemia and myelodysplasia. This IRF-1 tumour suppressor inactivation is caused by accelerated exon skipping of IRF-1 mRNA. The exon skipped form of *IRF-1* lacks exons 2 and 3 displaying neither DNA binding or tumour suppressor activity; thus this exon skipping may cause the inactivation of *IRF-1* (Harada *et al*., 1994).

Other pathological effects of IRF-1 mutation have been reported. Moriyama *et al* (2001) reported that IRF-1 expression was reduced in 53% of human hepatocellular carcinomas with both wild-type p53 and reduced expression of p21. Further, loss of heterozygosity on the long arm of chromosome 5 has been reported to be associated with stomach adenocarcinoma (Tamura *et al*., 1996). One of the targets of 5q loss of heterozygosity in colorectal carcinoma is certainly the adenomatous polyposis coli (*APC*) gene on 5q21. However, other evidence suggested the presence of another tumour suppressor gene within that area that might be inactivated in gastric carcinoma. Minimum regions of deletions including the *IRF-1* locus have been identified in gastric carcinoma using nine microsatellite loci (Tamura *et al*., 1995). Further study revealed this deletion in *IRF-1* tumour suppressor activity in gastric carcinoma is due to missense mutation in exon 2 where the ATG to TTG mutation caused a methionine to leucine substitution at amino acid 8 (Nozawa *et al*., 1998). Such a substitution allows mutant IRF-1 to bind to DNA with similar affinity as the wild-type protein, but abolishes its ability to induce interferon-β p125 luciferase promoter or inhibit cell growth when overexpressed (Nozawa *et al*., 1998).

1.4.2 Induction of IRF-1

IRF-1 mRNA is expressed at a low basal level in all cell types with the exception of early embryonic cells (Kroger *et al*., 2002). IRF-1 mRNA levels accumulate in response to many stimuli, including viruses, lipopolysaccharide (LPS), interferon, double stranded RNA, cytokines (IL-1, IL-2, IL-6, IL-12) and some hormones such as prolactin (Miyamoto *et al*., 1988; Harada *et al*., 1989; Barber *et al*., 1995; Galon *et al*., 1999; Coccia *et al*., 2000; McAlexander; Yu-Lee 2001). All these factors are part of a signalling network that indicates the presence of infection and their induction of IRF-1 is an important host-defence mechanism. Interferon-γ is the strongest IRF-1 stimulus, and other combinations of stimuli, such as interferon-γ and tumour necrosis factor (TNF-α), induce even higher levels of IRF-1 mRNA (Kroger *et al*., 2002).

Further, IRF-1 expression can be induced in response to DNA damage (Pamment *et al*., 2002). Other molecules can antagonize IRF-1 induction, as is the case with IL-4, which reduces interferon-γ stimulated IRF-1expression (Coccia *et al*., 2000).

IRF-1 mRNA levels are cell-cycle regulated (Stevens *et al*., 1992), with mRNA levels increasing in serum-starved cells in G_0 and decreasing following serum-induced growth. In addition, IRF-1 mRNA levels increase before and during S phase of the cell cycle (Kroger *et al*., 2002), confirming the short half-life of IRF-1 mRNA. IRF-1 can also activate the IRF-2 promoter, whereas IRF-2 inhibits the transcription of IRF-1 induced genes acting as a negative-feedback mechanism to limit post-induction IRF-1 activity.

IRF-1 is mainly induced using interferon alpha/beta receptor (IFNARs) receptor compartment using JAK and STAT pathways (section 5.2.1). In addition to this, IRF-1 has been reported to be induced by nuclear factor kappa B (NF-κB) signalling, where binding of the p50/p65 heterodimer form of NF-κB to the IRF-1 promoter region has been shown to stimulate IRF-1 expression and co-acts with NFκB to stimulate other downstream targets (Moschonas *et al*., 2008).

Finally, IRF-1 stimulation by DNA damage is induced through the ataxia telangiectasia mutated (ATM) signalling pathway, where DNA-damage induced IRF-1 was only reported when ATM is present (Pamment *et al*., 2002).

1.4.3 Function of IRF-1

IRF-1 was first recognized for its role in viral response; however, recently IRF-1 was known for its tumour suppressor activity. In order to link these two functions together it is important to understand the concept of "immune surveillance of tumours" (Burnet and Thomas 1957). This concept proposes that the immune system can specifically identify and eliminate tumour cells on the basis of their expression of certain tumour-specific antigens (Swann and Smyth 2007). However, this hypothesis of Burnet and Thomas (1957) was under question until recently when new data clearly show the existence of cancer immune surveillance and also indicated that it may function as a component of a more general process of cancer immunoediting (Dunn *et al*., 2002).

Evidence to support the immune surveillance of tumours includes the fact of spontaneous tumour development in immunodeficient mice as reported by Shankaran *et al* (2001) and Smyth *et al* (2000). In addition, carcinogen-induced tumours in immunodeficient mice also provided a great tool to study immune surveillance of tumours (Engel *et al*., 1997 and Svane *et al*., 1996).

Further on, a number of clinical observations have provided evidence supporting the idea of tumour immune surveillance in humans. The increased risk of tumour development in immunosuppressed patients (Penn 1988), instances of spontaneous tumour regression (Buckowitz *et al*., 2005), and the appearance of tumour-reactive T-cells and B-cells in relation to improved prognosis all point to a role for the immune system in suppressing tumour growth (Scanlan *et al*., 2004).

This following section explains IRF-1's role in the immune system, cell growth and apoptosis linking all three areas together as these functions are not totally separated; however, they all help toward understanding IRF-1's role in tumour suppressor activity. Following stimulation of the IRF-1 pathway, IRF-1 can initiate immune response (1.4.3.1), inhibit cellular proliferation (1.4.3.2), or initiate programmed cell death "apoptosis" (1.4.3.3).

1.4.3.1 The immune response and IRF-1

The host defence against extracellular pathogens is a notable example of a complex cellular response coordinated by a genetic regulatory network in which a given transcription factor controls the expression of a diverse set of target genes. Notable example of such case is the host defence against extracellular pathogen which is based on such alteration in the genetic regulatory network. The innate and adaptive immune systems are tightly connected within each other. As explained before, IRF-1 was discovered as a regulator of innate immunity through the interferon system suggesting a link between these two immune systems.

Following microbial invasion and interferon stimulation, IRF-1 protein levels get elevated to initiate the host defence mechanism. It has been shown that IRF-1 in wild-type macrophages, induced by interferon-γ and/or lipopolysaccharide, can form a complex with IRF-8 and bind ISRE at the regulatory element of *iNOS* resulting in the production of nitric oxide (Taniguchi *et al*., 1997); however, this complex formation between IRF-1 and IRF-8 does not occur in IRF-$1^{-/-}$ macrophages (Kamijo *et al*., 1994). This nitric oxide enzyme plays a vital role in the effector phase of the T-helper 1 (T_H1) response, which causes macrophage cytotoxicity against tumour cells, bacteria and other invading bodies through DNA damage (Wink *et al*., 1991). Thus, IRF-1 may be a master gene playing a role in T_H1 immune response.

In addition, IRF-1 has been reported to play a major role in the transcriptional activation of the *IL-12 p35* gene (Liu *et al*., 2003). IL-12 is a heterodimeric cytokine consisting of p40 and p35 chains together; however, these two chains are encoded by two separate genes on two separate chromosomes. Coordination of expression of these two genes is crucial for an appropriate immune response in macrophages induced by interferon-γ following pathogen stimulation. Evidence showed IRF-1 deficient macrophages have impairment in mRNA synthesis of IL-12 p35 but not p40 and a strong deficiency in the production of IL-12 p70 but not p40 (Liu *et al*., 2003). In addition, the same group reported that IRF-1 physically interacts with an inverted IRF element within the IL-12 p35 promoter after interferon-γ activation (Liu *et al*., 2003).

Additionally, IRF-1 has been reported to initiate the induction of type I interferon (interferon-α and -β). Overexpression of IRF-1 in human fibroblasts, IRF-1 induction by poly (I:C) treatment or Newcastle disease virus infected fibroblast cells expressed higher levels of interferon-β mRNA and protein than control cells (Reis *et al*., 1992). Recently Yarilina *et al* (2008) reported that tumour necrosis factor (TNF) initiated an interferon-β mediated autocrine loop that maintained the expression of inflammatory genes and delayed the expression of other interferon response genes such as *STAT1* and *IRF-7* resulting in an enhancement of the macrophage response to stimulate cytokines and Toll-like receptors. This autocrine loop is found to be regulated and depends on IRF-1 (Yarilina *et al*., 2008).

IRF-$1^{-/-}$ mice were also found to have defective thymocyte development and the same team observed that IRF-1 is required for the development of the T_H1 type immune response, and that its absence leads to the inappropriate induction of the T_H2 type immune response (Taniguchi *et al*., 1997). Confirming this observation, IRF-$1^{-/-}$ mice were also found to be vulnerable to bacterial infection when compared to IRF-1 wild-type mice (Taniguchi *et al*., 1997); however, the immune response appeared to be normal in $p48^{-/-}$ mice suggesting the unique role of IRF-1 in causing such immune effects (Taniguchi *et al*., 1997).

1.4.3.2 The tumour suppressor activity of IRF-1

After discovery IRF-1 was described as a nuclear transcriptional factor that regulates interferon inducible genes and plays a role in the immune response. Further, mouse fibroblasts were shown to be arrested in the G_1 phase of the cell cycle after culturing them in the absence of serum. Re-addition of serum stimulates these cells to continue through the cell cycle. However, during the G_1 arrest as a result of serum starvation, the level of IRF-1 mRNA is remarkably raised. Following the re-addition of serum, the amount of IRF-1 mRNA declined 6-fold (Harada *et al*., 1993). The same study also showed that overexpression of IRF-2 in mouse fibroblasts caused cellular transformation and enhanced tumorigenicity in nude mice; this observation was reversible as a result of IRF-1 overexpression (Harada *et al*., 1993). IRF-1 can also reverse the transformation of fibroblasts following the overexpression of other oncogenic proteins such as c-Myc or FosB (Tanaka *et al*., 1994). These finding suggested the first anti-oncogenic and oncogenic potential of IRF-1 and IRF-2 where restraining cellular growth depends on a balance between these two mutually antagonistic nuclear factors (Harada *et al*., 1993).

The knock-down of endogenous IRF-1 in human myeloid leukaemia cell lines stimulates cell proliferation where IRF-2 levels remained unchanged (Sato *et al*., 1997). This notable cellular proliferation occurs following STAT1 and STAT3 phosphorylation during interferon-γ pathway stimulation (Sato *et al*., 1997). In addition, Yim *et al* (2003) reported a similar observation in human breast carcinoma and concluded that growth inhibition is initiated with IRF-1 overexpression where such an effect can be overcome once IRF-2 is overexpressed.

Overexpression of IRF-1 using recombinant adenoviral vector (Ad-IRF-1) in mouse breast cancer cell lines resulted in apoptosis using the involvement of caspases (Kim *et al*., 2004); in addition, they also reported the suppression of growth for breast cancer cell lines *in vivo* by intratumoral injection of Ad-IRF-1 into established tumours in their natural host (Kim *et al*., 2004).

The same group also reported that ectopic expression of IRF-1 promotes breast cancer cell death *in vitro* which has turned to be associated with the down-regulation of servivin, an inhibitor of apoptosis protein (Pizzoferrato *et al*., 2004). Resected tumours from IRF-1 treated mice showed IRF-1 positive and servivin negative cells (Pizzoferrato *et al*., 2004); however, the mechanism by which IRF-1 suppresses servivin is still unknown.

Other studies have associated the induction of IRF-1 with cell cycle arrest. Tanaka *et al* (1996) reported the cooperation of the tumour suppressor IRF-1 and p53 in response to DNA damage. In mouse embryonic fibroblasts lacking IRF-1, cells are deficient in their ability to undergo DNA-damage induced cell cycle arrest as the cells lack p53 (Tanaka *et al*., 1996). Although IRF-1 and p53 expression is independent of each other but their cooperation take place in inducing p21, a cell cycle inhibitor, after cells are induced by γ-irradiation (Tanaka *et al*., 1996). Both IRF-1 and p53 can bind to p21 promoter directly or indirectly where both transcription factors regulate the cell cycle through the activation of certain cell cycle genes. In clinical trials as an attempt to treat patients with malignant midgut carcinoids using interferon-α, inhibition of cell proliferation via cell cycle arrest in G_1-S phase is mediated by the induction of p21 and IRF-1 (Oberg 2000). Direct binding of IRF-1 onto the IRF binding element of the p21 promoter has been reported (Coccia *et al*., 1999) and the ability of IRF-1 to activate the p21 promoter independently of p53 during DNA damage has also been shown (Tanaka *et al*., 1996).

Other examples of IRF-1 acting as an anti-oncoprotein includes the use of a lung carcinoma cell line where infecting the cells with measles virus results in IRF-1 up-regulation and thus G_0/G_1 cell cycle arrest (Yokota *et al*., 2004). A similar observation was noticed in transformed fibroblasts, where the activation of IRF-1 induces G_1 cell cycle arrest in *c-myc* and *ras* expression NIH3T3 (Kroger *et al*., 2007).

Taken together the finding in these studies, the tumour suppressor role of IRF-1 and its role in mediating cell cycle arrest through the induction of p21 has been supported. This role of IRF-1 and p21 in cell cycle arrest may be an important element in the suppression of cancer growth.

1.4.3.3 IRF-1 in programmed cell death

The first role of IRF-1 in apoptosis was reported by Tanaka *et al* (1994). They reported that transformation of embryo fibroblasts requires the introduction of mutated oncogenes such as *c-ras* and *myc*. However, in wild-type fibroblasts, containing wild-type p53 and IRF-1, cells undergo apoptosis following the introduction of mutated *c-ras* and DNA damage (Tanak *et al*., 1994). However, suppression of apoptosis was noticed in both $p53^{-/-}$ or $IRF\text{-}1^{-/-}$ embryo fibroblast cell lines (Tanaka *et al*., 1994).

Further, interferon-γ induced apoptosis in interferon-γ sensitive ovarian cancer cells was reduced after IRF-1 was knocked down or IRF-2 overexpressed (Kim *et al*., 2002). In addition, a recent study showed that overexpression of IRF-1 can enhance apoptosis in gastric cancer cells by the aid of 5-fluorouracil (Gao *et al*., 2012). Further, IRF-1 has been reported to regulate both immune cell apoptosis and autophagy in a murine endotoxemia model (Zhang *et al*., 2012). They also reported decreased apoptosis and increased autophagy in IRF-1 knock-out mice (Zhang *et al*., 2012).

To confirm the role of IRF-1 in apoptosis, IRF-1 has been shown to bind directly to the promoter of many pro-apoptotic genes and thus initiates the process of apoptosis. The following section will cover some of these pro-apoptotic genes. Caspases are cysteine aspartic proteases that play essential roles in apoptosis, necrosis, and inflammation by proteolysis of proteins essential for cell survival (Alnemri *et al*., 1996; Vermeulen *et al*., 2005). Liu *et al* (2012) reported that overexpression of IRF-1 enhanced glomerular mesangial cell's apoptosis where knock-down of *IRF-1* revealed the opposite effect. The study was carried out using microarray analysis where results showed IRF-1, caspase-3 and caspase-8 up-regulation in mesangioproliferative glomerulonephritis mouse model, an inflammatory kidney disease in children; they also reported enhanced caspase-8

promoter activity by IRF-1 (Liu *et al*., 2012). A further study showed that IRF-1 can up-regulate targeted PUMA, a p53 up-regulated modulator of apoptosis which is known to activate apoptosis by the intrinsic pathway (Gao *et al*., 2009). It has been shown that IRF-1 binds to unique sites within the promoter of *PUMA* and activates PUMA transcription in cancer cells (Gao *et al*., 2009). One further IRF-1 target is *TRAIL* (tumour necrosis factor-related apoptosis inducing ligand), which induces apoptosis through binding to death receptors (Almasan *et al*., 2003). Results showed that retinoid-induced IRF-1 is required for TRAIL induction by both retinoic acid and interferon-γ (Clarke *et al*., 2004), and that exposure of breast cancer cells to both retinoic acid and interferon-γ enhances TRAIL promoter activity by IRF-1 (Clarke *et al*., 2004). Further, IRF-1 forms a signalling feedback loop with IRF-7, type I interferon, and STAT1 that regulate TRAIL in HIV-1 infected macrophages (Huang *et al*., 2009). In addition, the X-linked inhibitor of apoptosis protein (XIAP)-associated factor 1 (XAF1), which is a novel tumour suppressor and interferon stimulated gene sensitizing cancer cells to apoptosis, is also regulated by IRF-1 in gastrointestinal cancer (Wang *et al*., 2009).

1.4.4 Structure and function of human IRF-1

The human *IRF-1* gene consists of 10 exons, is 325aa in size, and, as shown previously, the mutated form of IRF-1 has been associated with cancer and many other disorders. Studies based on deletion mutants have helped in mapping the IRF-1 domains. The following section will highlight the different domains in IRF-1 structure and their distinct regulatory roles.

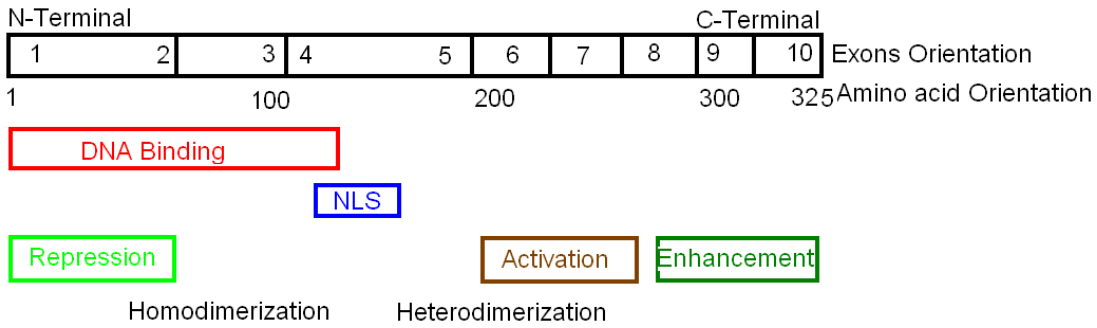


Figure 1.2: An outline of domain orientation and exon length in human IRF-1. The diagram shows the gene structure and domain orientation of IRF-1, which consists of 10 exons coding for a protein of 325 amino acids long. Important domains are mapped to their corresponding areas. NLS: nuclear localization domain.

1.4.4.1 The DNA binding domain

As shown in Figure 1.1, IRFs share a significant homology between their DNA binding domains (DBD). IRF-1 DBD is located in the first 124aa and characterized by five conserved tryptophan repeats separated by 10 to 18aa from each other. These tryptophan repeats mediate IRF-1 binding to the ISRE within the regulatory elements of target genes (Schaper *et al*., 1998).

1.4.4.2 The N-terminal transrepressor

The transrepressor domain in IRF-1 is located in the first 60aa and strongly inhibits its transcriptional activity. This fragment is conserved among other IRF members including IRF-2, IRF-3, and IRF-8; however, the corresponding sequence in IRF-9 is different so it does not show this inhibitory activity. Within the transrepressor domain of IRF-1, replacing the unique sequence in IRF-1 with its corresponding sequence in IRF-9 resulted in a loss of inhibitory activity within IRF-1 (Kirchhoff *et al*., 2000).

1.4.4.3 The homodimerization and heterodimerization domain

IRF-1 homodimerization domain is located within amino acids 90-115, where the heterodimerization domain span the area of 125-219aa. This domain is important for IRF-1 heterodimerization with activators and repressors of transcription. In addition, the two-hybrid system revealed that this domain aids in IRF-1 homodimerization *in vivo* (Kirchoff *et al*., 1998).

1.4.4.4 Nuclear localization domain

By using IRF-1 mutants fused to the green fluorescent protein and monitoring their distribution in living mammalian cells, the IRF-1 nuclear localization domain was mapped to amino acids 117–141 (Schaper *et al*., 1998).

1.4.4.5 Transactivation domain

A detailed deletion analysis of IRF-1 revealed the transactivation domain of IRF-1. The amino acid segment 217–260aa was necessary and sufficient for transactivation with a core activation domain of amino acids 233–255 (Kim *et al*., 2003). In addition, computer analysis predicated this region to be loop-helix-loop-sheet, and both the casein kinase II and protein kinase C phosphorylation sites on each of the two loops are not important for transactivation (Kim *et al*., 2003). Deletion of this core region interfered with wild-type IRF-1's ability to stimulate interferon-β expression, induce apoptosis, and inhibit cellular growth, possibly by forming inactive homodimers with wild-type IRF-1 or competing for target promoters (Kim *et al*., 2003).

1.4.4.6 Enhancer domain

A further fragment acts as a strong enhancer element located at the C terminus of IRF-1 at position 257–325 (Kirchhoff *et al*., 2000). A further enhancer subdomain was identified at an exact area of amino acids 301-325 (Eckert *et al*., 2006). The latter has been shown to regulate IRF-1 activity by inducing interferon-β genes and mediates the repression of cyclin dependent kinase II (*Cdk II*) (Eckert *et al*., 2006). In addition, the exact area of *Cdk II* repression was narrowed down to amino acids 306–310 (Eckert *et al*., 2006).

Studies performed within the enhancer area revealed that deletion of the last 70 amino acids inhibits both IRF-1 poly ubiquitination and degradation (Pion *et al*., 2009); however, deleting the last 25 amino acids (ΔC25 mutant) inhibits degradation of IRF-1 but does not prevent its ubiquitination (Pion *et al*., 2009). It is known that IRF-1 has a very short half-life (20–30min) (Pion *et al*., 2009) compared to IRF-2 (6–8hr). IRF-1 stability is controlled by the last 39 amino acids, as truncation of more than the 39 C-terminal amino acids generates a form of IRF-1 that could resist degradation by the ubiquitin-proteasome pathway (Nakagawa *et al*., 2000).

1.4.5 Regulation of IRF-1

As shown previously, IRF-1 expression is a tightly regulated process during the cell cycle and infection. IRF-1 can undergo post-translational modification for different biological roles including heterodimerization, degradation, and activity. These post-translational modifications include phosphorylation, ubiquitination, SUMOylation, and dimerization with other proteins or co-factors to respond to a different range of stimuli such as DNA damage, interferon, and, Toll-like receptor activation. The following section will highlight each modification in details.

1.4.5.1 IRF-1 phosphorylation

The role of phosphorylation on IRF-1 regulation is not fully characterized. However, Lin *et al* (1999) reported a kinase activity that co-purified with IRF-1 and IRF-2. The kinase activity was identified as two molecules with 43 and 38kDa molecular weight. Further biochemical studies revealed that these molecules belong to the alpha catalytic subunit of casein kinase II (CKII). In addition, Western blot analysis and Immunoprecipitation of protein–protein interactions demonstrated that this kinase interacted directly with IRF-1 at two cluster sites (Lin *et al*., 1999). These sites are located between amino acids 138–150, where the other is located toward the C terminus between amino acids 219–231 (Lin *et al*., 1999). Mutational studies within these two clusters reported a decrease in IRF-1 transactivation ability suggesting that CKII phosphorylation is important for regulating IRF-1 function. Finally, Sharf *et al*. (1997) reported IRF-1 phosphorylation by tyrosine kinase following interferon-γ stimulation.

1.4.5.2 IRF-1 ubiquitination

Proteolysis or protein degradation is an important cellular process to maintain the quality of the cellular proteome. In eukaryotic cells, many proteolysis systems are available, and among all these, the ubiquitin-proteasome system (UPS) is the most characterized. UPS is a critical cellular process where proteins get tagged with poly-ubiquitin chain and targeted for degradation by the 26S-protease (Sorokin *et al*., 2009). The process involves the covalent attachment of a small protein (76aa) ubiquitin (Ub) to a chosen protein substrate (Sorokin *et al*., 2009). The conjugation of Ub to the chosen substrate occurs via a three-stage cascade reaction: the first stage involves an enzyme termed E1 (Ub-activating enzyme), which activates ubiquitin in an ATP dependent manner, enabling its transfer into the second enzyme termed E2 (Ub-carrier enzyme) (Sorokin *et al*., 2009). The third enzyme is E3 (Ub-protein ligase) transfers Ub from E2 onto a lysine residue of the substrate protein. After connecting the first Ub onto targeted protein, E3 attaches another Ub molecule to the first attached Ub as poly-ubiquitination is required for degradation.

The E3 substrate recruiting machinery catalyzes the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of lysine residue on the substrate (Sorokin *et al*., 2009). The minimum signal for degradation by the proteasome is a chain of four Ub molecules (Thrower *et al*., 2000).

IRF-1 degradation is mediated by poly-ubiquitination, as inhibiting the proteasome activity caused an increase of a poly-ubiquitinated form of IRF-1 (Nakagawa *et al*., 2000). In addition, the ΔC25 mutant of IRF-1 inhibits the proteasome degradation of IRF-1 but does not prevent its ubiquitination (Pion *et al*., 2009). Within that site, an LXXLL motif that is required for binding of heat shock protein 70 (Hsp70) family members, and cooperation with Hsp90 to regulate IRF-1 activity has been identified (Narayan *et al*., 2009). Therefore, Hsp70 binding to IRF-1 could regulate and mediate IRF-1 degradation using UPS. Further on, CHIP (C terminus of Hsc70-interacting protein) was identified as a chaperone of IRF-1 under stressed conditions (Narayan *et al*., 2011). CHIP binds directly to the IRF-1 central domain (residues 106–140), leading to IRF-1 ubiquitination reduction and suggesting a role for CHIP as an E3 ligase (Narayan *et al*., 2011).

1.4.5.3 IRF-1 SUMOylation

SUMOylation is another form of protein post-translational modification. Covalent modification of cellular proteins by small ubiquitin-like modifiers (SUMO) regulates various cellular process including cell cycle, nuclear localization, signal transduction, and stress response (Muller *et al*., 2001); however, SUMOylation is differ from ubiquitination as SUMOylation does not tag target protein for degradation but it enhances their stability or modulates their subcellular compartmentalization (Muller *et al*., 2001).

Like ubiquitination, all SUMO forms are initially inactive forms and the carboxyl-terminal proteolysis cleavage event results in exposing the carboxyl-terminal glycine residue to allow isopeptide bond formation between the carboxyl terminus of SUMO with the ε-amino group of lysine residue on the substrate (Muller *et al*., 2001). Following SUMO activation, a cascade reaction similar to ubiquitination is required to modify the target substrate.

First IRF-1 SUMOylation was reported in 2002 by Nakagawa and Yokosawa. They reported the isolation of an IRF-1 binding protein (using the yeast two-hybrid system), which was identified as protein inhibitor of activated STAT3 (PIAS3). PIAS3 was found to bind to SUMO-1 and ubiquitin-conjugating enzyme 9, which is an E2 in the SUMO-1 conjugating system. Co-expression of PIAS3 induces SUMO-1 modification of IRF-1 and represses IRF-1 transcriptional activity (Nakagawa and Yokosawa 2002). Thus PIAS3 functions as a SUMO-1 ligase for IRF-1 resulting in repression of IRF-1 transcriptional activity.

A similar yeast two-hybrid screen reported the purification of the SUMO-conjugating enzyme Ubc9, which interacts with IRF-1 and IRF-8 (Kim *et al*., 2008). The interaction between IRF-1 and Ubc9 was confirmed *in vitro* and *in vivo* and reported to inhibit IRF-1 transcriptional activity in a dose-dependent manner (Kim *et al*., 2008).

A further study showed that levels of SUMOylated IRF-1 are elevated in tumour cells and its tumour suppressive function is reduced (Park *et al*., 2010). They also reported that SUMOylated IRF-1 mimics IRF-2 and displays oncogenic characteristics, as transfection of SUMOylated IRF-1 into nude mice resulted in tumour formation and infiltration of adipose tissue (Park *et al*., 2010).

1.4.6 Co-factors of IRF-1

1.4.6.1 NF-κB

Complex formation between IRF-1 and nuclear factor kappa-B (NF-κB) has been reported in many different cell types. Sanceau *et al* (1995) reported that the induction of IL-6 by interferon-γ and tumour necrosis factor-α (TNF-α) in monocytic cells involves the cooperation between IRF-1 and the NF-κB p65 subunit (Sanceau *et al*., 1995) or the p50 subunit (Neish *et al*., 1995). In addition, Saura *et al* (1999) reported both IRF-1 and NF-κB co-localize in the nucleus in TNF-α and interferon-γ stimulated macrophages, where complex formation between these two transcriptional factors occurs only in stimulated cells following binding of their target promoter site (Saura *et al*., 1999). A further study reported a similar cooperation in T-cells following HIV-1 infection (Sgarbanti *et al*., 2008). This functional form of IRF-1 and NF-κB occurs at the long terminal repeat kappa sites.

1.4.6.2 STAT1

IRF-1 has been shown to interact with the STAT1 transcription factor. Chatterijee-Kishore *et al* (2000) reported this interaction is required to mediate the transcription of low molecular mass polypeptide 2 (LMP2). The interaction site on IRF-1 to mediate this binding was mapped to amino acids 170–200 (Chatterijee-Kishore *et al*., 2000). In addition, the adenovirus E1A was reported to block the interaction between IRF-1 and STAT1 (Chatterijee-Kishore *et al*., 2000), resulting in the down–regulation of LMP2. Recently, Wang *et al* (2010) showed that the cooperation between the IRF-1 and STAT1 transcriptional complex mediates interferon-γ induced oligodendrocyte progenitor cells.

1.4.6.3 IRF-8

IRF-1 and IRF-8 have been reported to compete for ISRE binding; thus, IRF-8 has been shown to have a repressive effect on IRF-1 activity (Weisz *et al*., 1994). IRF-8 has been shown to bind directly to IRF-1 and thus reduces IRF-1 ability to bind to other co-factors such as HIV protein trans-activator of transcription (TAT) (Sgarbanti *et al*., 2002).

Further, the IRF-1/IRF-8 complex has been shown to transcriptionally activate *IL-12* p35 (Liu *et al*., 2004) and *RANTES* (Liang *et al*., 2006). Recently, Zhang *et al* (2010) reported IL-27 p28 gene transcription is cooperated between IRF-1 and IRF-8, as IRF-8 deficient macrophages were highly defective in IL-27 p8 expression. They also reported that IRF-1 and IRF-8 bind overlapping regions located at 48 to 57 of IL-27 p28 promoter regions *in vivo* following stimulation on interferon-γ (Zhang *et al*., 2010).

1.4.6.4 p300

Mouse IL-12 promoter activity has been reported to be stimulated by the cooperation of IRF-1 and IRF-8 transcriptional complex (Masumi *et al*., 2002); however, this promoter stimulation can be enhanced when p300 is co-transfected (Masumi *et al*., 2002). Further, Dornan *et al* (2004) reported that p300 binding to IRF-1 stimulates p53 acetylation. It was also revealed that stimulation of p21 transcription by IRF-1 does not require its DNA binding activity, but is based on the ability of IRF-1 to bind to p300 and stimulate p53 dependent transcription (Dornan *et al*., 2004).

1.4.6.5 PCAF

The p300/CBP-associated factor (PCAF) is a human gene and transcriptional activator associated with many other transcriptional factors (Ge *et al*., 2009; Fuks *et al*., 1998; Jin *et al*., 2002). DNA affinity binding assays of nuclear extracts showed that endogenous IRFs bound to ISRE cooperate with PCAF (a histone acetyl transferase) and p300 binding proteins (Masumi *et al*., 1999). PCAF transfection strongly enhances IRF-1 and IRF-2 promoter activity (Masumi *et al*., 1999).

1.4.6.6 NPM

Nucleophosmin (NPM) is a nuclear phosphoprotein that shuttles between the nucleus and cytoplasm and regulates ribosomal RNA export from the ribosome (Maggi *et al*., 2008). NPM was identified as an IRF-1 binding protein (Kondo *et al*., 1997), and functional analysis showed NPM inhibits the DNA binding and transcriptional activity of IRF-1 (Kondo *et al*., 1997). NPM was classified as an oncogene as its mRNA levels were found to be elevated in several leukaemia samples and its overexpression can transform mouse embryonic fibroblasts (Kondo *et al*., 1997).

1.4.6.7 GRIP-1

Glucocorticoids (GC) are a class of steroid hormones used in managing patients with asthma (Bhandare *et al*., 2010) their name is derived from its role in glucose regulation, adrenal cortex synthesis location, and their steroidal chemical structure. However, asthma patients can develop GC insensitivity, which represents a challenge for asthma management. Such resistance is meditated by the mutual inhibition of transcriptional activity between GC receptor and other regulators such as IRF-1 (Bhandare *et al*., 2010). IRF-1 has been found to promote insensitivity in human airway smooth muscle cells by interfering with GC receptor signalling (Tliba *et al*., 2008), this effect of IRF-1 was found to be caused by the cooperation of IRF-1 with GC-receptor interacting protein 1 (GRIP-1) (Bhandare *et al*., 2010). Co-immunoprecipitation and GST pull-down showed GRIP-1 forms a transcriptional complex with IRF-1.

1.4.6.8 MyD88

Myeloid differentiation primary response gene 88 (MyD88) is an adapter protein recruited by Toll-like receptors after microbial and pathogen stimulation of the immune response. It has been shown that MyD88 recruits members of IRFs including IRF-5 and IRF-7 (Honda *et al*., 2004 and Takaoka *et al*., 2005) to activate Toll-like receptor target genes. In addition, IRF-1 also has been shown to interact with MyD88 upon Toll-like receptor activation (Negishi *et al*., 2006). IRF-1 has been shown to migrate into the nucleus more efficiently once associated with MyD88 as a part of Toll-like receptor dependent gene induction programme (Negishi *et al*., 2006).

1.4.6.9 KPNA2

Karyopherin α-2 (KPNA2) is an important protein playing a role in signal transduction between the cytosol and nucleus during the course of keratinocyte proliferation and differentiation (Umegaki *et al*., 2007). It has been shown that interferon-γ down regulates KPNA2 expression in normal human epidermal keratinocytes at both mRNA and protein levels at the promoter level (Umegaki *et al*., 2007). In addition, KPNA2 has been found to physically bound to IRF-1 and induce nuclear translocation of IRF-1 in normal human epidermal keratinocytes (Umegaki *et al*., 2007).

1.5 Conclusion and thesis aims

All the previous studies mentioned in this chapter indicated that IRF-1 can function as a part of a transcriptional complex, and that disrupting this complex can influence IRF-1 regulation and activity. The studies described here support the theory that IRF-1 protein–protein interaction and IRF-1 post-translational modification play an important role in controlling IRF-1 activity and function. However, most of these studies were based on transiently transfecting IRF-1 and the use of artificial models for most of their findings. It will be interesting to develop synthetic tools that can aid in designing a more physiologically relevant micro-environment in which transcriptional factors can be studies at their local environment. Such tools will aide in understanding the mechanism of cancer initiation and progression and speed up cancer drug screening to identify new potential anti-cancer drugs. The research conducted in this thesis is primarily aimed at the development of specific molecular tools that can be used to study transcriptional factors and other gene of interests at their physiological relevant environment.

Chapter 2: General methods and techniques

2.1 Chemicals and reagents

All chemicals were ordered from BDH Laboratory Supplies or Sigma-Aldrich unless otherwise stated in the thesis. Tissue culture medium and supplements were obtained from Gibco (Invitrogen), tissue culture plastics and other plasticware were obtained from TPP or Nunc. All oligonucleotides were synthesized by Sigma-Genosys. All restriction enzymes were supplied by New England Biolabs.

2.2 Mammalian cell line

A375, an epithelial like human malignant melanoma derived from a primary melanoma lesion of a 54 years old female patient. A375 was used throughout the project; A375 cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum (Autogen Bioclear) and 1% (v/v) penicillin-streptomycin mix (P/S). Cells were maintained 10% CO_2 at 37°C in incubators supplied by Hera.

2.2.1 Mammalian cell line sub-culturing

When cells were approximately 80–90% confluent, the medium was discarded and cells were washed three times with sterile phosphate-buffered saline (PBS), followed by a short trypsinisation with 0.05% Trypsin-EDTA at 37°C. Fresh medium was added to trypsin to deactivate it and to make the required dilution, followed by seeding the cells onto fresh plates containing fresh medium.

2.2.2 Freezing and thawing cells from/for liquid nitrogen storage

Cells were frozen at 70–80% confluence to assure viability of the cells. Cells in 10cm tissue culture plates were washed and trypsinised as described above and transferred into 15ml falcon tube and spun down at 1000rpm for 5min at room temperature. The medium was then discarded and cells were resuspended in 10ml freezing medium (50% v/v FBS, 40% v/v DMEM, and 10% v/v DMSO).

The mixture was transferred into cryogenic vials and placed in a cryo-freezing container (Nalgene) at -80°C overnight to ensure gradual freezing. Lastly, cells were transferred to liquid nitrogen for long-term storage.

Liquid nitrogen frozen cells were thawed as fast as possible. Cells were quickly placed at 37°C until almost all cells were thawed, then cells were added to a 15ml flacon tube containing fresh 9ml of DMEM. Cells were spun down at 1000rpm for 5min and the supernatant was discarded. Cells were resuspended in 10ml fresh medium and transferred to a sterile culture dish. The medium was replaced after 24hr to boost cells growth.

2.2.3 Mammalian cell line transient transfection

Cells were transfected using Attractene transfection reagent (Qiagen) as described in the manufacturer's handbook. Briefly, cells were sub-cultured into a10cm tissue culture plate 24hr before transfection. 4µg of desired DNA, 15µl of Attractene, and FBS and P/S-free DMEM were all mixed together in an Eppendorf tube to make a total volume of 300µl. Mixture was left at room temperature for 15min then poured onto a 10cm plate.

2.2.4 Mammalian cell line treatment

In order to induce required responses, cells were treated with certain reagents. In all cases, cells were seeded onto suitable tissue culture plates 24hr before treatment. The following day, cells were washed twice with PBS and then cultured in medium containing poly (I:C), interferon-γ, kinase inhibitor, or tunicamycin (table 2.1) for a specific time period, as outlined specifically in each chapter. Following treatment, cells were washed twice with PBS and harvested as outlined in section 2.2.1 and 2.7.1.

Reagent	Function	Diluent	Stock Concentration	Company
Poly (I:C)	Mimic viral infection	Water (-20°C)	2.5mg/ml	Sigma-Aldrich
Interferon-γ	Induce IRF-1 pathway	40mM Tris + 0.1% BSA (pH 7.4) (-80°C)	200units/ml	Gibco
Kinase inhibitor	Target kinase activity	DMSO (-20°C)	200mM	Tocriscreen™
Tunicamycin	ER stress	Water (-20°C)	1µg/ml	Sigma-Aldrich

Table 2.1: Reagents used in cell treatment

However, in case of UV treatment, medium was removed and adherent cells were exposed to UV dosage using a UV box, followed by recovery of treated cells by replacing fresh medium on the plate. UV dosage and recovery time is indicated in experimental details in results chapter(s).

2.3 Microbiological techniques

2.3.1 Bacterial strains

DH5α chemo-competent *E. coli* cells were used as the standard strain for routine propagation of bacterial plasmids including entry vectors and recombined destination vectors. SW102 is another recombineering competent *E. coli* strain, which was used as a host for recombineering cloning. Both strains were grown aerobically in liquid culture or on solid agar in order to obtain single bacterial colonies.

For liquid culture, bacterial cells derived from a single colony were inoculated in LB broth (10g tryptone, 5g yeast extract, and 10g NaCl, all dissolved in 1litre distilled water and sterilized by autoclaving). Antibiotics were added to the media as required, to a final concentration of: ampicillin 100µg/ml; kanamycin 50µg/ml; chloramphenicol 34µg/ml.

Bacterial cells were incubated at 37°C or 32°C on an orbital shaker for at least 4hr to a maximum of 18hr. For liquid cultures, the culture vessel was at least five times the volume of the medium to ensure proper aeration.

For growth on solid media, bacterial cells were spread on LB-agar plates (10g tryptone, 5g yeast extract, 10g NaCl, 15g sucrose, all dissolved in 1litre distilled water and sterilized by autoclaving). LB-agar plates were prepared by pouring liquefied LB-agar into 90mm petri dishes (Sterilin). When required, selective antibiotics were added to the liquefied LB-agar, immediately prior pouring into petri dishes, which was cooled until solidified. Plates were then dried at 37°C for up to 1hr prior to use. Bacteria were grown on agar plates at 37°C or 32°C for 18hr.

2.3.2 Bacterial glycerol stocks

Bacteria cultures were stored long-term at -80°C in cryovials containing 200µl sterile glycerol and 800µl of an overnight liquid culture. Stocks were mixed by gentle agitation before storage at -80°C.

2.3.3 Heat-shock competent bacterial cell preparation

Glycerol stocks of the required *E.Coli* bacterial strain were inoculated into 3ml LB medium and allowed to grow overnight at 37°C. 250µl of the overnight culture were transferred into 50ml LB and incubated at 37°C until the $O.D._{600nm}$ of the culture reached approximately 0.4. Cells were collected by centrifugation at 4000rpm for 15min at 4°C, resuspended gently in 16ml ice-cold buffer 1, pH5.8 (1.2g RbCl, 0.99g $MnCl_2 \cdot 4H_2O$, 0.59g CH_3COOK, 1.5g $CaCl_2 \cdot 2H_2O$ and 15% v/v glycerol in 100ml distilled H_2O) and left on ice for 10min. Then the cells were centrifuged again at 4000rpm for 15min at 4°C and gently resuspended in 2ml ice-cold buffer 2, pH6.8 (10mM MOPS pH6.8, 0.12g RbCl, 1.1g $CaCl_2 \cdot 2H_2O$ and 15% v/v glycerol in 100ml distilled H_2O). After 10min incubation on ice, the cells were snap-frozen as aliquots in liquid nitrogen and stored at -80°C.

2.3.4 Recombineering competent bacterial cells preparation

Glycerol stocks of the required SW102 bacterial strain were inoculated into 3ml LB medium and allowed to grow overnight at 32°C. 500µl of the overnight culture were transferred into 35ml LB and incubated at 32°C until the $O.D._{600nm}$ of the culture reached approximately 0.4–0.6. Cells were split into two flasks: one was allowed to continue growing at 32°C for further 15min and the second was allowed to continue growing at 42°C for further 15min to induce recombineered protein expression. Cells were then rapidly chilled on ice-water slurry for 10min. Cells were collected by centrifugation at 4000rpm for 7min at 4°C. The pellet was washed with ice-cold distilled water three times. The final washed pellet was resuspended with 200µl sterilized ice-cold water. Recombineering competent cells were made freshly every time they were used and not subjected to long-term storage.

2.3.5 Bacterial transformation (heat-shock method)

An aliquot (50µl) of heat-shock competent cells was incubated on ice with an appropriate amount of plasmid (usually 50ng) for 30min. The cells were then subjected to heat-shock by incubating at 42°C for 45sec, and then left at room temperature for 10min. LB medium was added to the competent cell/plasmid mixture and cultured for 1hr at 37°C or 32°C to allow the growth of transformed bacterial cells. Finally cells were plated onto pre-prepared LB-agar plates with selection by antibiotics specific for the plasmid transformed. Plates were incubated at 37°C or 32°C overnight for colony formation.

2.3.6 Bacterial transformation (electroporation method)

An aliquot (50µl) of competent cells with the desired amount of DNA (usually 0.1ng) was placed in a 2mm electroporation cuvette (Molecular BioProduct). Cuvettes were placed in a Gene Pulser MXcell Electroporation system (Bio-Rad) pre-set to 1.80kV, 25µF capacitance and 200Ω resistance. Following electroporation, LB medium was added to competent cell/plasmid mixture and cultured for 1hr at 32°C to allow the recovery of transformed bacterial cells.

2.3.7 Plasmids and BAC maintenance

After transformation, single colonies were selected and allowed to grow overnight either in 5ml LB medium for mini-prep or 150ml LB medium for maxi-prep. To purify plasmids from culture, mini-preps or maxi-preps were carried out using the high speed mini-prep or maxi-prep kits from Qiagen following the manufacturer's protocols. However, BAC mini-prep involved a slight adjustment to the Qiagen protocol, in which single colonies carrying BAC DNA were allowed to grow overnight in 5ml LB medium followed by pelleting 1.5ml of overnight culture by centrifugation in a 1.5ml tube at 10,000rpm at room temperature for 1min. 100µl of Qiagen buffer I was used to suspend the pellet, followed by ice incubation for 5min. 200µl of Qiagen buffer II was added to the suspended cells, followed by gentle mixing and ice incubation for a further 5min. 150µl of Qiagen buffer III was also added to the mixture followed by gentle mixing and ice incubation for a further 5min. The whole mixture was centrifuged at 10,000rpm for 5min at 4°C. DNA from the supernatant was then ethanol precipitated by adding two volumes of 95% ethanol and placed on ice for 30min. Following incubation, the mixture was centrifuged at 10.000rpm for 10min at 4°C. Pellet was washed with 70% ethanol several times and air dried followed by resuspension in 30–50µl of distilled water. The concentration of BAC DNA and plasmids after purification was measured using an ND-3300 Fluorospectrometer (NanoDrop Technologies), at 260nm. Purified DNA was stored at -20°C.

2.4 DNA and mRNA assays

2.4.1 mRNA extraction

mRNA was extracted from cells and tissues using the Qiagen RNeasy Mini kit following the manufacturer's suggested procedures. Briefly, cells were lysed and homogenized using highly denaturing guanidine-thiocyanate buffer resulting in RNase inactivation to ensure an intact RNA purification. Ethanol was then added to provide suitable RNA binding condition allowing total RNA binding to the membrane of the RNeasy Mini spin column where contaminants were washed away; RNA was then eluted in 50µl water. The optional step of DNase treatment using the Qiagen RNase-free DNase set was also included. The concentration of the extracted mRNA was measured using the ND-3300 Fluorospectrometer (NanoDrop Technologies) at 260nm.

2.4.2 Reverse Transcription (RT) Polymerase Chain Reaction (PCR)

Reverse transcription of mRNA was performed using the Omniscript RT kit from Qiagen following the manufacturer's suggested protocols. Briefly, RNA from section 2.4.1 was incubated with reverse transcription reaction buffer (10x), dNTP mix (5mM), oligo-dT primer (10µM), RNase inhibitor (10units/µl), and 4units of Omniscript reverse transcriptase in a 20µl total volume reaction. Mixture was incubated at 37°C for 60min. Reverse-transcribed cDNA was either used for standard PCR using *pfu* polymerase kit from Stratagene according to manufacturer's suggestions or for qRT-PCR; see next section.

2.4.3 Real-time PCR (qRT-PCR)

Real-time PCR or qRT-PCR was performed using the Solaris qPCR Gene Expression Assays (Dharmacon) following the manufacturer's suggested protocols. Briefly, mRNA was extracted as 2.4.1. 500ng of extracted RNA were reverse transcribed as 2.4.2 to generate cDNA, which was diluted to make a stock concentration of 25ng/µl. 125ng of cDNA, 6.25µl of PCR water, 1.25µl of Solaris primers (Dharmacon), and 12.5µl of Solaris master mix were all mixed together, each sample was treated in quadruplicate for statistical significance. Samples were placed in the qRT-PCR machine using the following thermal cycling program: initial step at 95°C for 15min (enzyme activation) then 40 cycles of 95°C for 15sec (denaturation) and 60°C for 60sec (annealing and extension).

2.4.4 Agarose gel electrophoresis

DNA samples were mixed with 6x DNA loading buffer (30% v/v glycerol, 0.6% w/v SDS, 60mM EDTA and 0.1% w/v bromophenol blue) before loading on the gel. Agarose gels were made by dissolving agarose in heated 1xTris/Borate/EDTA (TBE) buffer (90mM Tris, 90mM Boric acid and 2mM EDTA). The percentage of agarose in the gel depended on the size of the DNA to be separated. As a size reference, a DNA marker from New England Biolabs was loaded next to the DNA samples. Electrophoresis was performed at 100V until a good resolution was observed under a UV transilluminator. The images of the gels were captured using the ChemiGenius2 Bio-Imaging system from Syngene.

2.5 Genetic engineering and cloning

2.5.1 Gateway® Cloning

In order to clone PCR products into Gateway® plasmids, LR and BP cloning was used. A PCR product was amplified, using primers containing Gateway® sites, in a standard PCR reaction. Once amplified, the PCR product was cleaned using Qiagen PCR-clean up kit. BP Gateway® cloning kit from Invitrogen was used following the manufacturer's suggested protocols. To further clone the PCR product from pDONR221 into pEF5/FRT/V5-Dest, the LR Gateway® cloning kit from Invitrogen was used according to manufacturer's suggested protocols. The only adjustment to the manufacturer's protocol was the incubation period, where in both cloning (LR and BP) tubes were incubated for 24hr at room temperature.

2.5.2 DNA sequencing

DNA sequencing reactions were carried out using the BigDye Terminator v3.1 cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Automated sequencing was carried out by the DNA sequencing service in Genetics Core, University of Edinburgh, Western General Hospital, Edinburgh, UK. All sequencing primers used in this project are outlined in table 2.2.

Primer Name	Primer Sequence
3′ homologous arm check F	5′-AGCCTCTGTTCCACATACAC-3′
3′ homologous arm check R	5′-ATCTTCTTCCTCAGGGTTGC-3′
5′ homologous arm check F	5′-CATTGCCTCCTGGCTATTGG-3′
5′ homologous arm check R	5′-TAAGTCTGCAGGTCGAGGGACC-3′
M13 (Forward)	5′-GTAAAACGACGGCCAG-3′
T7 (Forward)	5′-TAATACGACTCACTATAGGG-3′
IRF-1 S1	5′-GGAGCCAGATCCCAAGACGTG-3′
IRF-1 S7	5′-CTCTTAGCATCTCGGCTGG-3′

Table 2.2: Sequencing primers used through project. Homologous arm check primers were used to confirm the integration of homologous arms in pLSL-TOPO-β-catenin arms (chapter 3), M13 and T7 are standard primers used to confirm successful LR and PB reactions, and finally IRF-1 S1 and IRF-1 S7 were used to sequence wild type IRF-1 and W11R mutant.

2.5.3 DNA digestion and ligation

Specific DNA digestion was achieved by DNA nucleases obtained from New England Biolabs. In general, 5units of each DNA nuclease were used to digest 1µg of DNA in a 10 or 20µl reaction at 37°C for a minimum of 1hr. Following digestion, enzyme was deactivated at 65°C for 20min. Some DNA nucleases required the use of BSA (bovine serum albumin) to increase enzyme efficiency. DNA ligation was achieved using T4 DNA ligase (New England Biolabs) where the reaction was always carried out in three different ratios of fragment:plasmid (1:1, 3:1, and 1:3); the reaction was carried at 16°C for 24hr.

2.5.4 Site direct mutagenesis

In order to create certain mutations on specific sites of plasmid, the Quickchange™ Site-Directed Mutagenesis kit from Stratagene was used, with the manufacturer provided protocols, to create IRF-1 W11R mutation (chapter 4). Primers for site-directed mutagenesis, as shown in section 4.2.2, were designed using the PrimerX software (Gao, 2003) http://www.bioinformatics.org/primerx/index.htm

2.6 Southern blotting

2.6.1 Southern blot immobilization

Once agarose gel was run to desired distance it was then soaked in 0.2M HCL for 10min. It was then rinsed once with distilled water and soaked in 0.4M NaOH and 0.6M NaCl for 30min. Immediately, the gel was placed on the blotting apparatus surrounded by 10x SSC (300mM sodium citrate, pH 7.0, and containing 1M sodium chloride) for an overnight immobilization on Biodyne® nylon membranes, as shown in figure 2.1.

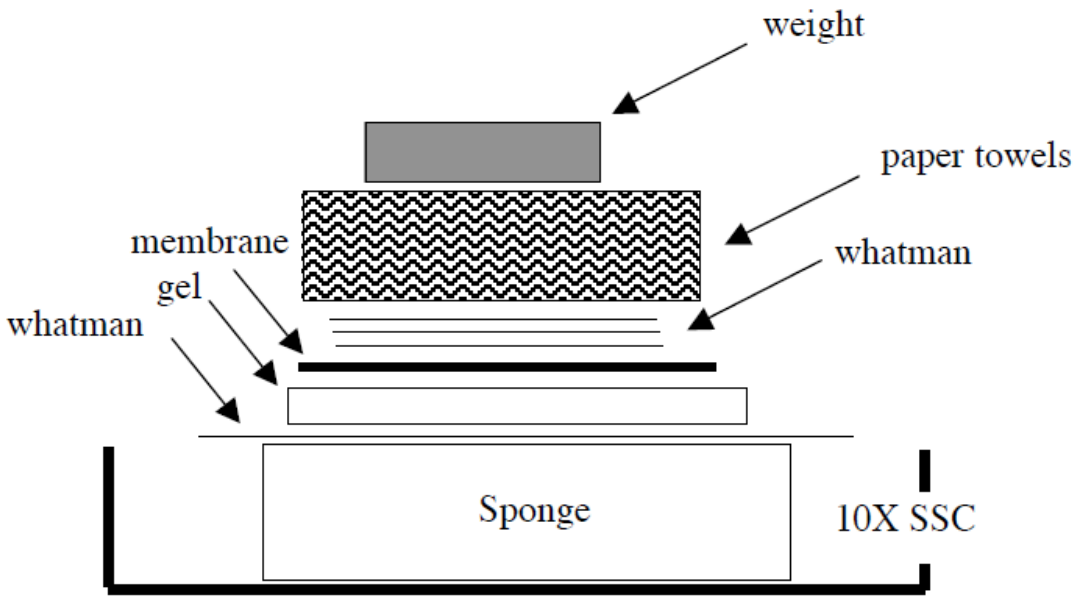


Figure 2.1: Blotting apparatus for Southern blots.

2.6.2 Southern blot probe hybridization

Following overnight immobilization, the membrane with immobilized DNA was washed with 6x SSC for 5min. The membrane was then placed in a heat-sealable bag, 5ml of prehybridization solution (Sigma) was poured onto membrane, air was removed, and the membrane was incubated for 2hr at 68°C. Following prehybridization, buffer was poured away and 5ml of hybridization buffer (Sigma) containing 100ng of radiolabeled denatured probe was poured onto membrane.

Probe hybridization took place at 58°C for 24hr. The following day, the membrane was submerged for 5min in a tray containing 200ml of washing buffer I (2x SSC and 0.5% SDS). The membrane was then transferred to a tray containing 200ml of washing buffer II (2x SSC and 0.1% SDS) and incubated for 15min at room temperature with gentle agitation. The membrane was then transferred into a further tray containing 200ml of washing buffer III (0.1x SSC and 0.5% SDS) and incubated for 30min at 37°C with gentle agitation. Membrane was then transferred into a tray containing 200ml of washing buffer III (0.1x SSC and 0.5% SDS) and incubated for 30min at 68°C with gentle agitation. Finally, the membrane was washed with washing buffer IV (0.1x SSC) and dried and wrapped to prevent contamination. Radiographic images were developed after exposing X-ray films to membrane for 24hr at -80°C.

2.7 Protein assays

2.7.1 Protein extraction from mammalian cells

In a typical experiment as outlined in 2.2.1, after being collected, the cells were spun at 2000rpm for 1min and the supernatant was removed. The cell pellet was washed 3 times with PBS, followed by lysis in approximately 4 volumes of Nonidet P40 (NP40) lysis buffer (1% v/v NP40, 150mM NaCl, 25mM Tris pH 7.5, 1mM Dithiothreitol (DTT) and 1x protease inhibitors). NP40 lysis buffer was applied to the cell pellet and left on ice for 30min followed by centrifuging at 12,000rpm for 15min. Supernatant was collected and pellet was discarded.

2.7.2 Protein concentration determination using Bradford

Bradford reagent was prepared by diluting a 5x Bradford stock solution (Bio-Rad). 1µl of protein sample was mixed with 200µl of Bradford reagent, mixed and incubated at room temperature for 5min to develop the colour reaction. The absorbance of the mixture was tested at 565nm using a PowerwaveXS Microplate Spectrophotometer (Bio-Tek). The protein concentration was calculated using a BSA standard curve.

2.7.3 Western Blotting

Western blotting was composed of two main steps: sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and immunoblotting. In SDS-PAGE, protein samples were first mixed with 4x SDS sample buffer (4% w/v SDS, 25mM Tris pH 6.8, 20% v/v glycerol, 0.01% w/v bromophenol blue and 200mM DTT) and incubated at 95°C for 2min before loading onto 8, 10, 12 or 15% polyacrylamide gel or 4–12% NuPAGE Bis-Tris gel (Invitrogen). The running gel was composed of 25% 1.5M Tris pH8.8, 0.1% SDS, 0.1% w/v Ammonium Persulphate (APS), 0.04% v/v Tetramethylethylenediamine (TEMED) and 41% of 30% w/v acrylamide mixture (National Diagnostics).

The stacking gel was composed of 25% 1M Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED and 17% of 30% w/v acrylamide mixture. Samples were resolved by electrophoresis in SDS running buffer (0.1% w/v SDS, 0.192M glycine and 25mM Tris) in a Bio-Rad mini-protean 3 apparatus. Samples on 4–12% NuPAGE Bis-Tris gels were resolved by electrophoresis in MOPS buffer (Invitrogen) in a Novex XCell SurelLock gel apparatus.

For immunoblotting, the resolved proteins were first transferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences) in transfer buffer (0.192M glycine, 25mM Tris and 20% v/v methanol) in a Bio-Rad transfer kit at 350mA for 2hr. An ice pack in the transfer tank was used to maintain low temperature to aid protein transfer.

Following transfer, the membrane was incubated in PBS-Tween-20 (0.1% v/v Tween-20 in PBS) (PBST) with 5% w/v dry skimmed milk (MARVEL) for 1hr at room temperature to block non-specific protein binding. After a brief wash with PBST, the membrane was incubated with the primary antibodies, at supplier-recommended dilutions in PBST with milk, and shaken at 4°C overnight (table 2.3). Blots were then washed three times, for 5min each, with PBST and incubated with species specific horse radish peroxidase (HRP) coupled secondary antibodies from DAKO in PBST with milk to detect specific antibody binding. Finally the blot was washed three times for 5min with PBST and incubated with electrochemiluminescence (ECL) solution mixture. ECL solution I was composed of 2.5mM luminol, 0.4mM coumaric acid and 100mM Tris pH 8.5, and ECL solution II composed of 100mM Tris pH8.5 and 0.018% v/v H_2O_2 (Bio-Rad). ECL solutions were kept in the dark at 4°C and mixed 1:1 prior to incubation with the blot. Specific bands were detected by exposing the membrane to ECL hyperfilm (Amersham Biosciences). Film exposure time varies between few seconds to one minute depending on the status of IRF-1. Interferon-γ or poly (I:C) stimulated IRF-1 can be detected within few seconds of exposure; however, endogenous un-stimulated IRF-1 requires a minimum of one minute exposure. In addition, DMSO has shown slight induction effect on IRF-1 (Figure 5.5); however, this effect was discarded as it was at very low level.

Antibody Name	Source	Company	Dilution
p53 antibody (DO1)	Mouse	In house antibody	1:1000
IRF-1 antibody (ARP38128_P050)	Mouse	BD Bioscience	1:1000
β-Actin antibody	Mouse	Sigma-Aldrich	1:5000
ISG-20 antibody (AB50834)	Rabbit	Abcam	1:1000
AGR-2 antibody (K47)	Rabbit	In house antibody	1:1000
PKR antibody (N-18)	Mouse	Santa Cruz Biotechnology	1:1000
p21 antibody	Mouse	In house antibody	1:500
CHOP antibody	Rabbit	Epitomics	1:1000

Table 2.3: Primary antibodies used in Western Blots.

2.8 Luciferase reporter gene assay

Reporter gene assays were performed using the Dual Luciferase® reporter system (Promega) according to the manufacturer's instructions. In brief, A375 FRT7, A375 FRT7 W11R, and A375 FRT7 conIRF-1 cells were seeded in 24-well plates 24hr before assay. The following day, cells were transiently co-transfected with pCMV-*Renilla*-luciferase (60ng) and 14ng of hTLR3-luciferase gene constructs. When required, the total amount of DNA in each well was normalized using empty vector control pcDNA3 (Invitrogen). Luminescence was measured for 100ms in a Fluoroskan Ascent F1 luminometer (Labsystems). Signals were normalized using an internal control. However, in the case of reporter stable cell lines, cells were seeded in 24-well plates 24hr before assay. The following day, cells were treated with a kinase inhibitor library, as outlined in chapter 5. The following day, luciferase activity was measured according to the manufacturer's instructions without normalizing to empty vector control.

2.9 Statistical methods

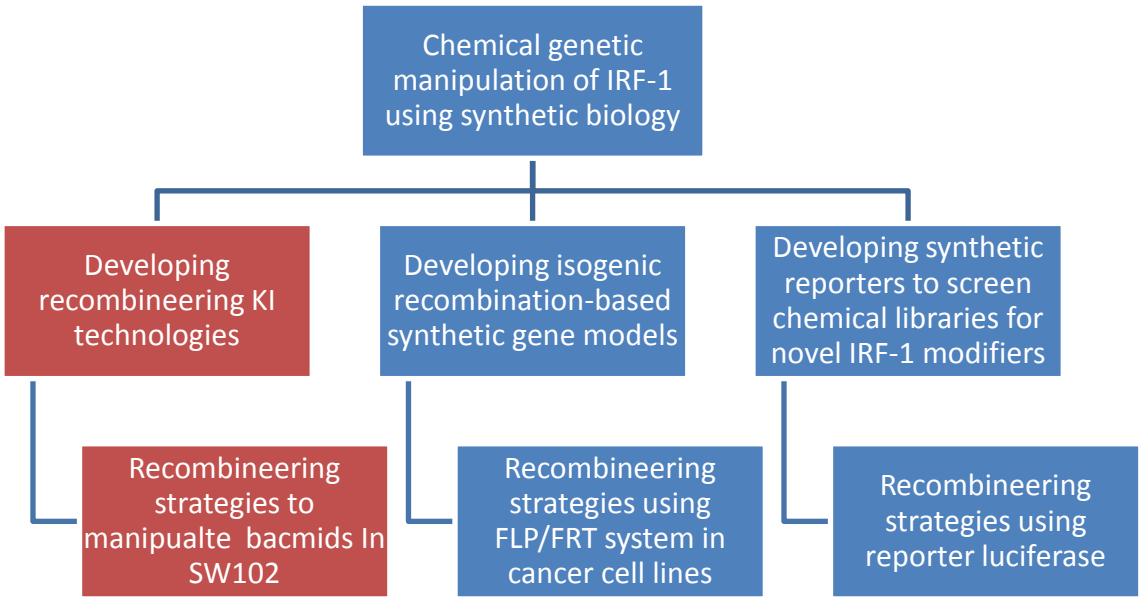
Microarray data analysis was done externally using (Fios Genomics, Edinburgh) raw data were QC analysed using the Array Quality Metrics package in Bioconductor (Kauffmann *et al*., 2009). Raw data were transferred using a Variance Stabilizing Transformation (VST) method prior to normalisation across all arrays using the Robust Spline Normalisation (RSN) method.

Dual Luciferase assay data analysis involved standard analysis where signal from the *Firefly* plasmid was normalized to signal from *Renilla*. Samples were done in triplicate for mean calculation and standard deviation error bars representation.

Chapter 3: Developing recombineering-knock-in technology

3.1 Chapter abstract

Cre-conditioned mouse models have become an essential requirement for any outstanding research project; however, generating a mouse model with a conditioned knock-in subtle mutation(s) has huge time and labour costs. This chapter outlines the generation, using recombineering technology, of a universal BAC targeting vector where a *cre*-conditioned STOP cassette can be targeted at a desired specific area. Also, different ways of creating these subtle mutation(s) on low/high copy plasmids have been discussed and outlined.



3.2 Chapter introduction

In 2003, the completed draft of the human genome was sequenced and annotated. The challenge now is to determine how these genes function, independently and with other genes in the human genome. The function of many genes is unknown and scientists need to characterize them to reveal their secrets. Mouse models became an ideal model system for such studies not only because humans and mice share a similar genome but also for many other reasons, which include availability and ease of handling.

Previously, any gene alternation, such as null mutation, subtle mutation, or gain of function mutation, could be introduced into the mouse germ line using traditional genetic engineering, using restriction endonucleases and DNA ligases, where the conditional inactivation of such genetic effects takes place only at certain tissue and/or certain developmental stages (Clarke, 2000). However, one big drawback in traditional genetic engineering is the difficulty of manipulating large linear DNAs; in addition, the need for complicated targeting and selection vectors is essential in this case where the production of such vectors is associated with the ability to find a unique and suitable restriction sites at vectors and genomic DNA. In most cases, this could be impossible as rare restriction sites occur frequently on large DNA sequences such as BACs and bacterial chromosomes (Copeland *et al*., 2001).

The development of systems based on homologous recombination had eased the process of generating transgenic and knock-out constructs, making it possible to manipulate large pieces of DNA, such as yeast artificial chromosome (YAC), BAC and P1 artificial chromosome (PAC), using different recombination systems in different recombineering hosts (Murphy 1998; Zhang *et al*., 1998; Yu *et al*., 2000; Muyrers *et al*., 2001). In 1993, Agnes Baudin reported the first homologous recombineering in yeast (Baudin *et al*., 1993); however, chromosome engineering in yeast (mainly *Saccharomyces cervisiae*) has a very efficient DNA double-stranded-break and-repair recombination pathway making it a good choice for manipulating mega sized genomic inserts.

However, yeast recombination is always active leading to unwanted genomic alteration. In addition, YACs are difficult to purify (Schedi *et al*., 1993) and single cell yeast could carry wild-type chromosome in addition to the modified YAC (Peterson *et al*., 1997).

To overcome these difficulties in yeast as a host for recombineering, *E. coli* was engineered to allow the manipulation of BACs. Unlike yeast, the presence of RecBCD, an ATP-dependant exonuclease that initiates recombinational repair acting as a helicase and a nuclease (Pilarksi *et al*., 1973), within the *E. coli* genome causes instability of linear dsDNA in *E. coli*. To overcome this problem, an *E. coli* strain lacking RecBCD, generated by mutating *recBC*, must be used; in addition to the *recBC* mutation, *sbcB* and *sbcC* mutations should also be achieved to restore recombination activity (Lloyd *et al*., 1985). However, recombination in *E. coli* RecBCD deficient strains will be limited, which restricts the use of *E. coli* for recombineering technologies. In addition, it has been reported that the recombination pathway is constitutively active, making continuous alterations and deletions in repeat sequences present in BACs and PACs (Copeland *et al*., 2001).

To protect linear dsDNA from degradation by RecBCD in wild-type cells, a Chi site (5′-GCTGGTGG-3′) is incorporated at each end of the linear dsDNA to protect from RecBCD digestion and provide activation sites on the DNA for RecBCD recombination (Dabert and Smith 1997). Once RecBCD encounters a Chi site, RecBCD loses its exonuclease activity but maintains its helicase activity leading to the production of ssDNA with a 3′ overhang end from the linear dsDNA, which allows recombination (Copeland *et al*., 2001). One big limitation of Chi sites in recombination is the fact that it is very inefficient (Copeland *et al*., 2001).

Stewart and his team (Zhang *et al*., 1998) have reported the possibility of using a phage encoded recombination system (RecET) using *recBC sbcA* strain where *sbcA* mutation activates the expression of *recE* and *recT* genes, which are encoded by a cryptic RAC prophage present in *E. coli* (Clark *et al*., 1994). Another recombination system is present in bacteriophage-λ, which is named as "Red". Like RecET, the Red system has two distinct genes, *redα* (*Exo*) and *redβ* (*Bet*), analogous to *recE* and *recT* (Carter and Radding *et al*., 1971). Exo (a 5′–3′ exonuclease) acts on linear dsDNA followed by Beta binding to the Exo-generated ssDNA overhang allowing strand annealing simulation (Figure 3.1) (Takahashi *et al*., 1990). The bacteriophage-λ encoded Gam inhibits any RecBCD activity in the host cell (Murphy 1991).

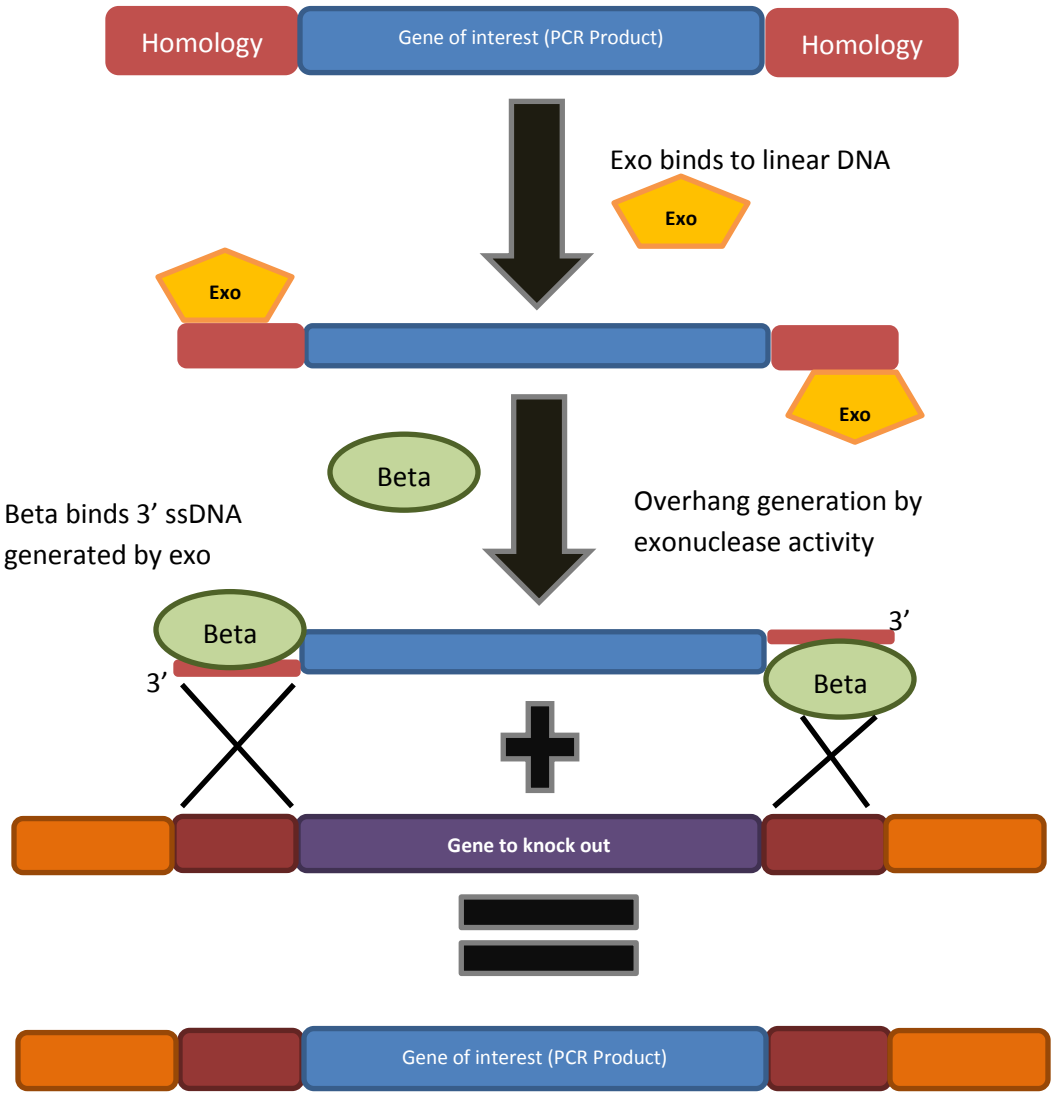


Figure 3.1: An overview of the recombineering system of bacteriophage-λ. Exo has a 5′ to 3′ exonuclease activity when bound to double stranded linear DNA resulting in 3′ single stranded overhang ends, which allows Beta to bind and promote single stranded annealing and recombinant DNA generation. This results in replacing the knock-out DNA with the gene of interest (PCR). In addition to Exo and Beta, a third protein named as Gam is needed to block the activity of RecBCD from degrading double stranded linear DNA in the host cell.

This system of bacteriophage-λ has been reported to be 10–100 times more efficient than any other recombineering system, such as *recBC*, *sbcBC* or *recD* strains (Murphy, 1998). The bacteriophage-λ system integrated in the *E. coli* chromosome has been modified to allow tight control of the expression of the recombineering proteins. Such modification involves the use of the *λ-cI857* repressor, which causes repression of recombineering protein gene expression at 32°C, making Exo, Beta, and Gam levels undetectable; however, when the culture is shifted to 42°C for 15min, the repressor become inactivated and the recombineering proteins are expressed at high levels under the control of the *λ-pL* promoter (Copeland *et al*., 2001). This modified bacteriophage-λ system has been transferred to BAC host *E. coli* strains such as DH10B to allow BAC recombineering (Lee *et al*., 2001). DH10B was later modified for higher efficiency creating another strain called DY380 (Lee *et al*., 2001). A further *E. coli* recombineering host strain is SW102; this host strain has a relevant genome to DY380 strain acquiring the bacteriophage-λ recombineering system. SW102 was chosen to be used in this project for its ability for *GalK* counter-selection (explained later in discussion).

To summarize, recombineering is adapted from homologous recombination, and it allows the precise insertion, deletion or even sequence alternation without the need for restriction sites or DNA ligases. Recombineering is a new *in vivo* method of genetic engineering that uses very short homologous arms, 20–70bp long (Copeland *et al*., 2001), with the aid of recombineering protein in a recombineering host, usually *E. coli*, to achieve the desired knock-in or knock-out (Sharan *et al*., 2009).

The aim of the work presented in this chapter was to create, using recombineering technology, a universal BAC targeting vector that can be used to create *cre*-conditioned knock-in mutations in any BAC of interest. The working plasmid template is named pLoxP-STOP-LoxP-TOPO (pLSL-TOPO; Addgene, plasmid number 11584). The main characteristic of this plasmid is the four polyA sites (stop cassette) flanked by *cre*-conditioned LoxP sites (Figure 3.2). However, the pLSL-TOPO to be used in BAC targeting, has to undergo certain modifications and adjustments, which are outlined next, to fulfil the requirement for BAC targeting using recombineering.

Currently at pLSL-TOPO stop cassette, there is a puromycin resistance sequence which is regulated by the eukaryotic PGK promoter to aid selection of transformed embryonic stem cells; however, it's not possible to use the puromycin sequence in prokaryotes unless a prokaryotic regulatory sequence, such as EM7, is cloned upstream of the sequence. In order to achieve this by recombineering, the whole puromycin sequence needs to be replaced by a new sequence containing EM7+kanamycin/puromycin; unfortunately, a prokaryotic regulated kanamycin sequence is already present in the plasmid backbone which will cause difficulties in selecting the desired recombined clones. Thus, the first step in modifying pLSL-TOPO was replacing the kanamycin sequence in the plasmid backbone with ampicillin (a good marker for selection in prokaryotes) followed by restoring kanamycin/puromycin resistance in the cassette. In addition, for BAC recombineering purposes, it is important to clone some unique restriction sites upstream of the LoxP-STOP-LoxP cassette to allow the conventional cloning of BAC recombineering homologous arms, which need to be done to allow the integration of the LoxP-STOP-LoxP cassette into the chosen BAC. Once the unique restriction sites have been cloned, the plasmid could be used universally for BAC targeting.

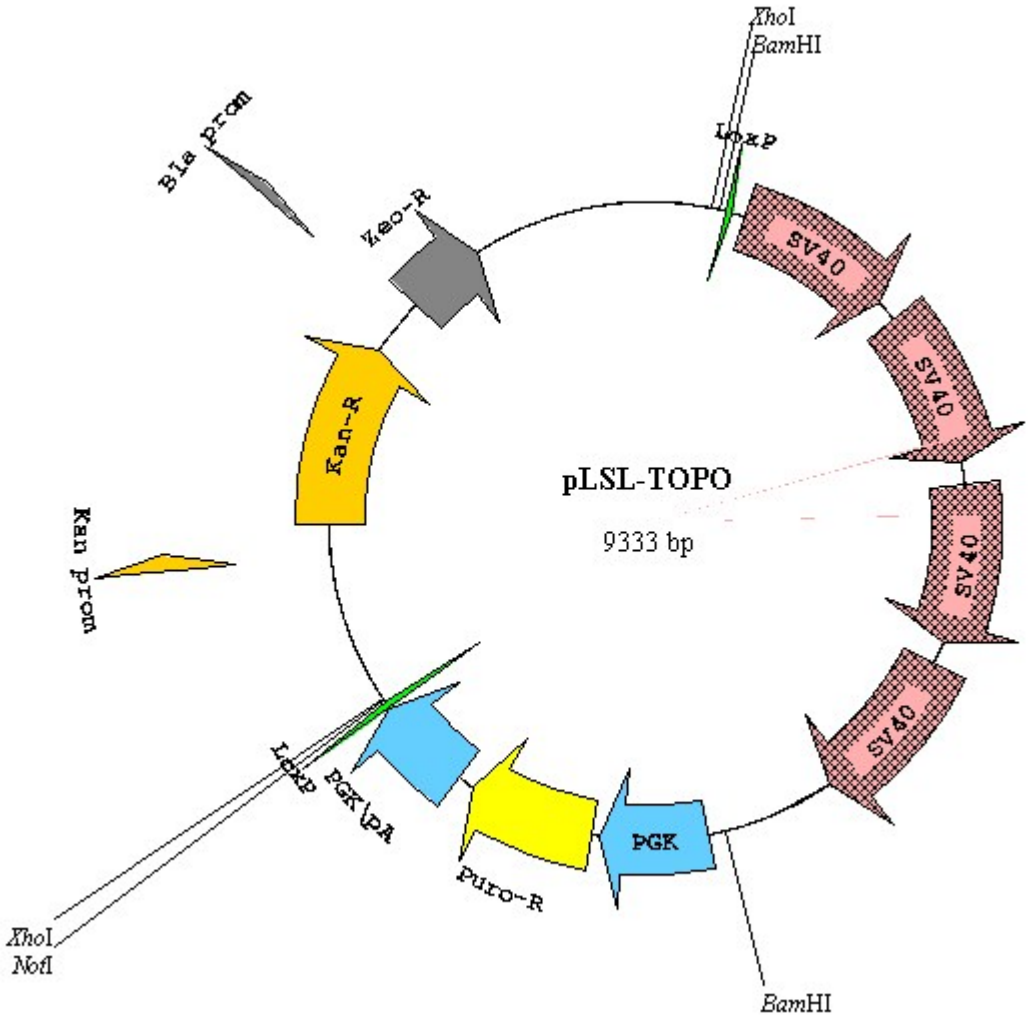


Figure 3.2: pLSL-TOPO (Addgene). The stop cassette consists of four SV40 polyA sites (pink), puromycin resistance gene (yellow) regulated by the eukaryotic PGK promoter (blue) flanked by two LoxP sites (green). In addition, two different antibiotic resistance sequences at backbone: Kan-R (orange) and Zeo-R (gray). Figure created using SimVector 4.5.

3.3 Methods and results

3.3.1 Replacing the original kanamycin resistance gene with an ampicillin resistance gene

On receipt of pLSL-TOPO (Addgene), the *E. coli* host strain was grown at 30°C to increase plasmid stability and yield. DNA was purified according to the miniprep protocol (Qiagen) and 50µg of plasmid DNA was digested by *NotI, BamHI* and *XhoI* to confirm the plasmid template and number of SV40 polyA sites agreed with the manufacturer's plasmid map (Figure 3.2) and (Figure 3.3.A).

PCR primers (green) with homologous arms (blue) were designed to amplify the ampicillin resistance gene (without the regulatory sequence) from plasmid pRosa26/*LacZeo* (kindly given by Dr. Peter Hohenstein).

Forward: 5′-ggcgcaggggatcaagctctgatcaagagacaggatgaggatcgtttcgcatgagtattcaacatttccgt-3′

Reverse: 5′-agatgcgtaaggagaaaataccgcatcaggaaattgtaagcgttaataatttaccaatgcttaatcagtga-3′

pRosa26/*LacZeo* was linearized by *XhoI* followed by *DpnI* digestion to minimise false colony formation post PCR reaction. Digested pRosa26/*LacZeo* (PCR template) was diluted 1:50, 1:100, and 1:500 and 1µl from each dilution was used in the PCR reaction. Each PCR cycle was performed at 95°C for 1min (denaturation step) followed by 56°C for 30sec (annealing step) followed by 72°C for 1min (elongation step) for 40 cycles. The PCR product was purified using a PCR clean-up kit (Qiagen) and size confirmed by separation on a 1% agarose gel (Figure 3.3.B). A combination of 20ng of pLSL-TOPO and a clean PCR product (100ng, 150ng, and 200ng) were then electroporated in heat induced and -un-induced (negative control) SW102 cells for recombination. Following recombination, cells were allowed to recover for 1hr at 32°C without antibiotic. Following the recovery hour, fresh LB containing ampicillin was added to the cells, which were grown overnight at 32°C. Plasmid was purified from tubes with positive growth by miniprep (Qiagen) and 0.1ng of plasmid DNA was re-electroporated into fresh SW102 to minimise the issue of copy number.

Potential clones were tested for their zeocin and ampicillin resistance and kanamycin sensitivity (Figure 3.3.C and 3.3.D). Clones were also digested with *KpnI* to confirm plasmid size to exclude any doubt of false-positive colony formation from pRosa26/*LacZeo* (PCR template) as *KpnI* will linearize both plasmids (pLSL-TOPO and pRosa26/*LacZeo*) into 9.3kb band 14kb band respectively (Figure 3.3.E). At this stage it was important to confirm the presence of the four SV40 polyA sites in the plasmid using *BamHI* digestion where a band of around 4kb confirms the intact of all 4 polyA sites (Figure 3.3.F). As this stage, the first version of the modified vector is named as pLSL-TOPO-Amp (Figure 3.4).

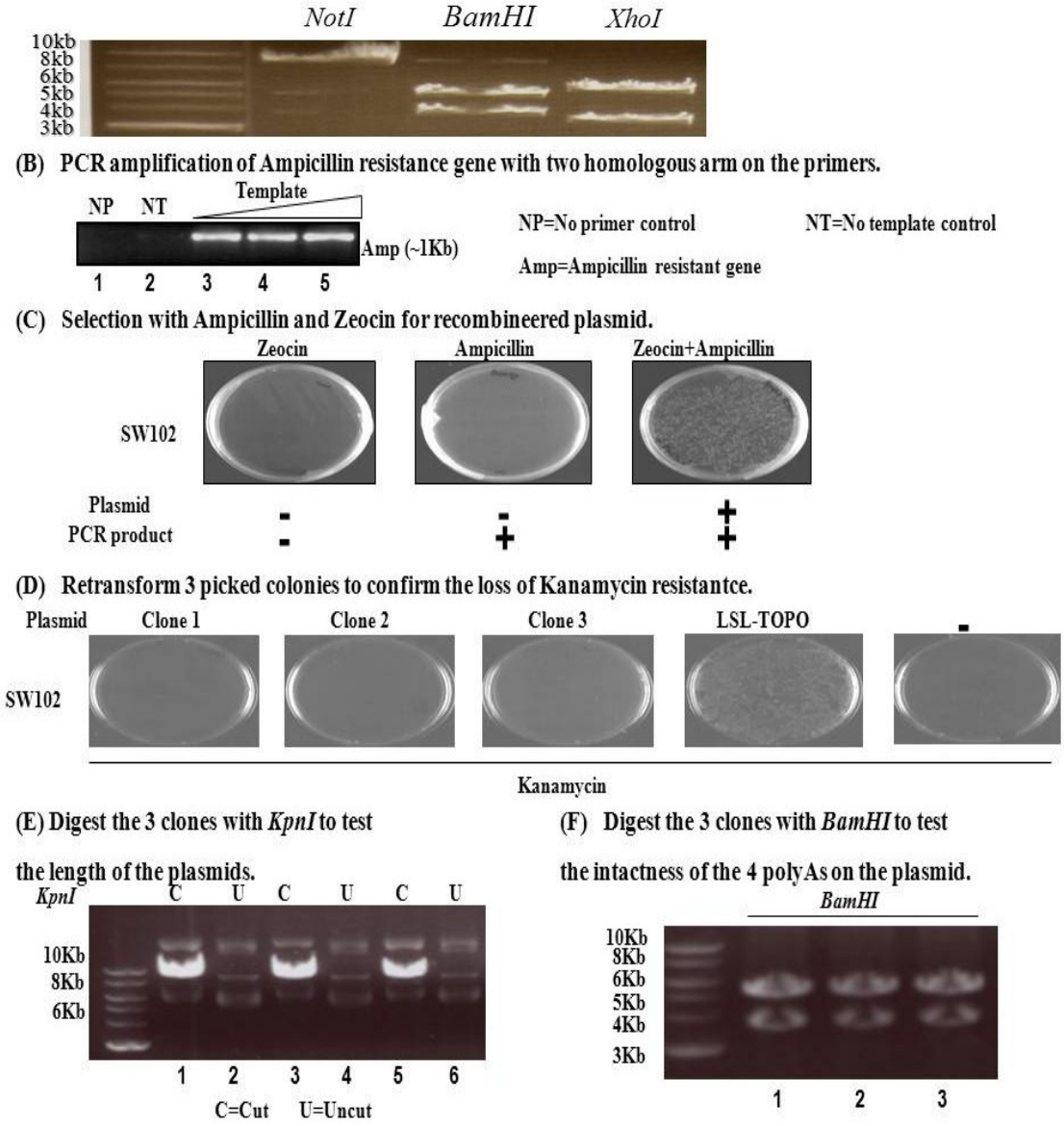


Figure 3.3: The first step in adapting pLSL-TOPO for BAC recombineering. (A) The integrity of pLSL-TOPO and 4 SV40 polyA sites were confirmed (B) Ampicillin resistance gene with homologous arms was amplified from pRosa26/*LacZeo* and electroporated into SW102 with pLSL-TOPO for recombineering. Colonies with successful recombineering are zeocin and ampicillin resistant (C) and kanamycin sensitive (D). (E) General plasmid integrity was confirmed by the presence of an 8kb band as a result of linearizing pLSL-TOPO-Amp (E) and verification that the four SV40 polyA sites are still intact (F).

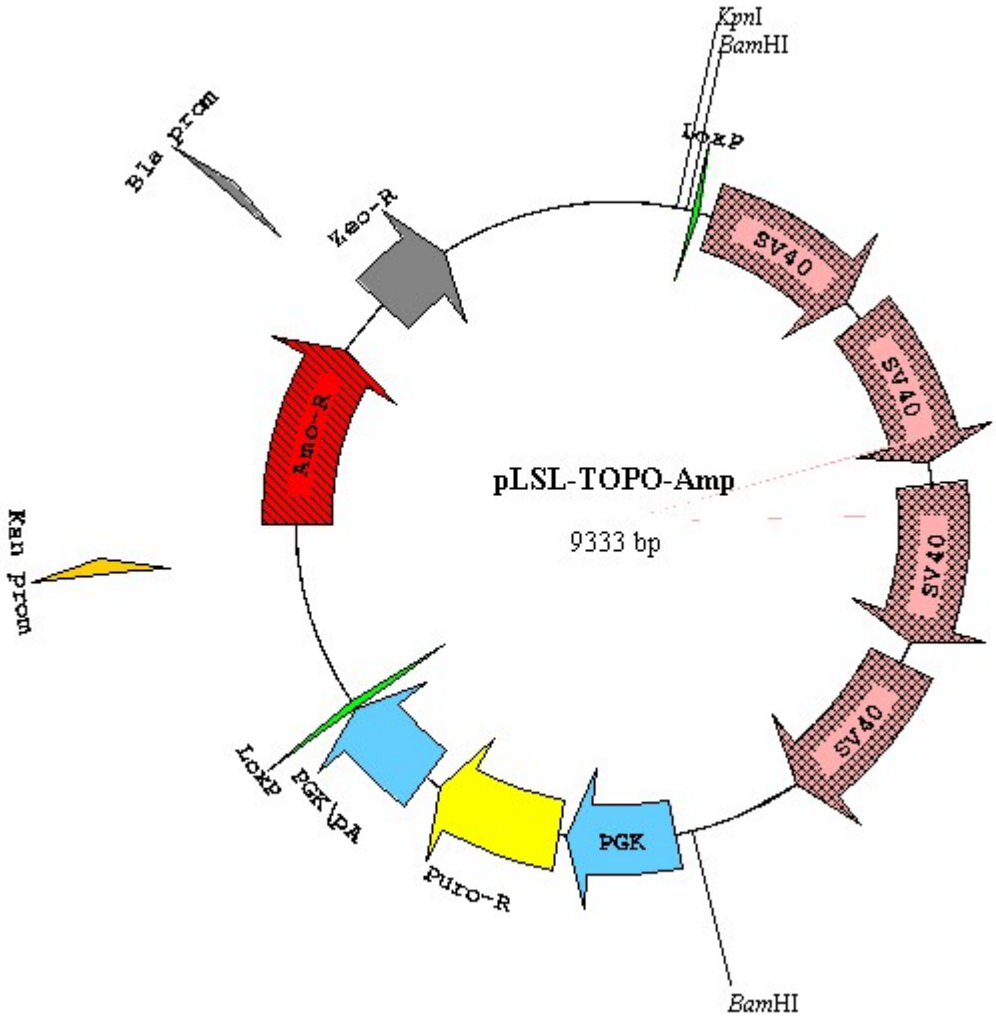


Figure 3.4: pLSL-TOPO-Amp. The plasmid map shows the ampicillin resistance gene instead of kanamycin (red), and the four intact SV40 polyA sites (pink). Figure created using SimVector 4.5.

3.3.2 Replacing the original puromycin resistance gene with the EM7 promoter and kanamycin resistance gene

Once pLSL-TOPO-Amp was created, it was necessary to restore the kanamycin/neomycin resistance gene under the control of the EM7 promoter. PCR primers (green) with homologous arms (blue) were designed to amplify the kanamycin/neomycin resistance gene with the EM7 regulatory sequence from a pLoxP-F3-neoF3 (kindly given by Dr. Peter Hohenstein).

Forward: 5′-cctttcgacctgcatccatctagatctcgatcgagcagctgaagcttaccggttgacaattaatcatcggc-3′

Reverse: 5′-tggggtcgtgcgctcctttcggtcgggcgctgcgggtcgtggggcgggcgtcagaagaactcgtcaagaa-3′

pLoxP-F3-neoF3 was linearized by *EcoRI* followed by *DpnI* digestion to minimise plasmid replication and false-positive colony formation post PCR reaction. Digested pLoxP-F3-neoF3 (PCR template) was diluted 1:500 and 1µl was used in the PCR reaction. Each PCR cycle was performed at 95°C for 1min (denaturation step) followed by 56°C for 30sec (annealing step) followed by 72°C for 1min (elongation step) for 40 cycles. PCR product was purified using PCR clean-up kit (Qiagen) and confirmed by separation on a 1% agarose gel (Figure 3.5.A). A combination of 20ng of pLSL-TOPO-Amp and a clean PCR product (100ng, 150ng, and 200ng) were then electroporated in heat induced and -un-induced (negative control) SW102 cells for recombination. Following recombination, cells were allowed to recover for 1hr at 32°C without antibiotic. Following the recovery hour, fresh LB containing ampicillin and kanamycin was added to the cells, which were grown overnight at 32°C. Plasmid was purified from tubes with positive growth by miniprep (Qiagen) and 0.1ng of plasmid DNA was re-electroporated into fresh SW102 cells to minimise the issue of copy number and plated on kanamycin-containing agar plates (Figure 3.4.B). Potential clones were digested with *NotI* and *PstI* to confirm the recombineering of EM7-Kan/Neo insert and to confirm the presence of the four intact SV40 polyA sites (Figure 3.5.C). As this stage, the second version of the modified vector is named as pLSL-TOPO-Neo (Figure 3.6).

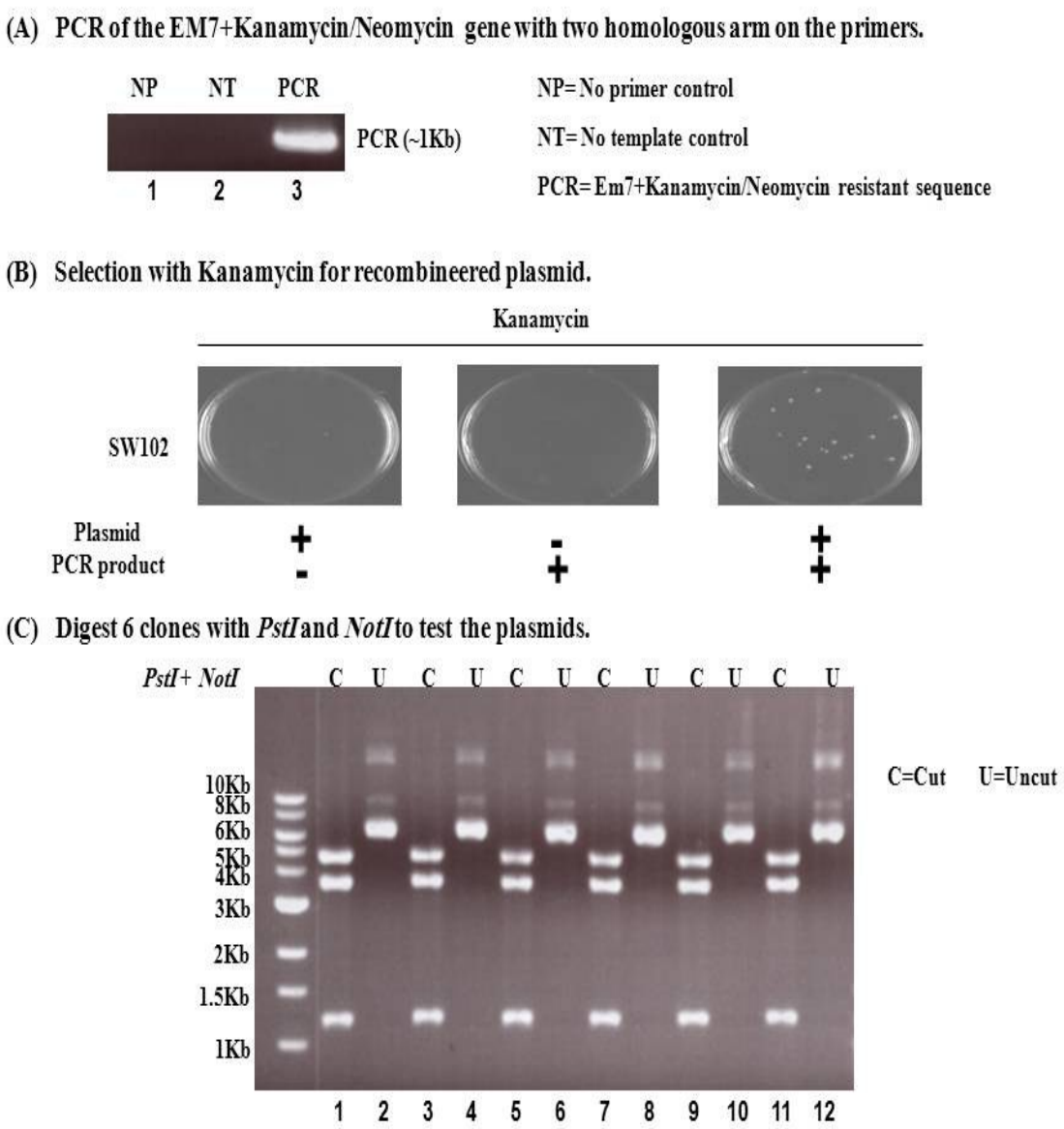


Figure 3.5: The second step in adapting pLSL-TOPO for BAC recombineering. (A) EM7 regulatory sequence + kanamycin/neomycin resistance gene with homologous arms was amplified from pLoxP-F3-neo-F3 and electroporated into SW102 with pLSL-TOPO-Amp for recombineering. (B) Colonies with successful recombineering are kanamycin resistance. (C) Kanamycin resistant clones were tested using *PstI* and *NotI* digestion which should result in 3 bands 1196bp, 3328bp, and 4779bb.

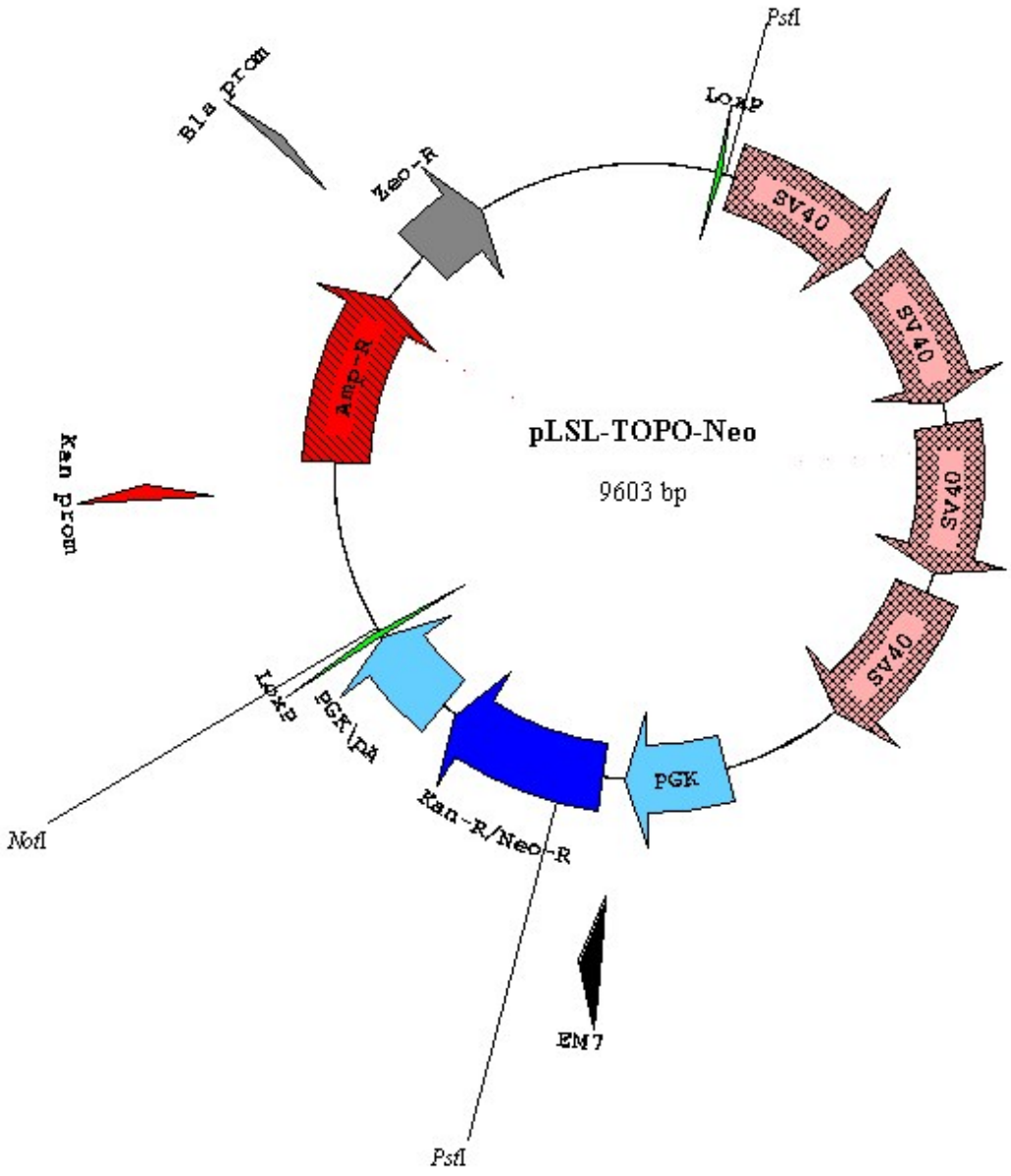


Figure 3.6: pLSL-TOPO-Neo. The plasmid map shows the new kanamycin/neomycin resistance gene (dark blue) and EM7 promoter (black), the four intact SV40 polyA sites (pink). Figure created using SimVector 4.5.

3.3.3 Cloning unique restriction sites downstream of the LoxP-STOP-LoxP cassette

In order to insert unique restriction sites downstream of the LoxP-STOP-LoxP cassette, *NotI* at pLSL-TOPO-Neo was chosen. The following oligonucleotides were designed in such a way that they will looses the *NotI* site at pLSL-TOPO-Neo once they become ligated following pLSL-TOPO-Neo digestion with *NotI*.

```
        AvrII   -   I-SceI   -   PmeI        -       I-CeuI       -       NdeI
5′ -GGCCCCTAGGTAGGGATAACAGGGTAATGTTTAAACCGTAACTATAACGGTCCTAAGGTAGCGAACATATG

    GGATCCATCCCTATTGTCCCATTACAAATTTGGCATTGATATTGCCAGGATTCCATCGCTTGTATACCCGG-3′
```

The ordered oligos were resuspended in water, boiled at 95°C for 5min and allowed to cool down at room temperature to permit sequence annealing for 3hr; serial dilution of (1:10, 1:100, 1:1000, and 1:10,000) were made. pLSL-TOPO-Neo was single digested with *NotI* and 1µl of each oligonucleotide dilution was ligated into *NotI* cut pLSL-TOPO-Neo by T4 DNA ligase. Following ligation, plasmid was re-cut again using *NotI* to cut any self ligated plasmid. 1µl of ligation mix was electroporated into un-induced SW102 and cells were allowed to recover for 1hr at 32°C without antibiotic. Following the recovery hour, fresh LB containing ampicillin and kanamycin was added to cells, which were grown overnight at 32°C. Plasmid was purified from tubes with positive growth by miniprep (Qiagen) and 0.1ng of plasmid DNA was re-electroporated into fresh SW102 cells and plated on kanamycin-containing agar plates (Figure 3.7.A). Potential clones were digested with *NotI* to confirm the loss of the *NotI* site (Figure 3.7.B). In addition, clones were digested with the new unique *NdeI* restriction site (Figure 3.7.C, left) and, to confirm the intact of four SV40 polyA sites, clones were also digested by *BamHI* (Figure 3.7.C, right). The third version named as pLSL-TOPO-BAC (Figure 3.8) of the modified pLSL-TOPO was created.

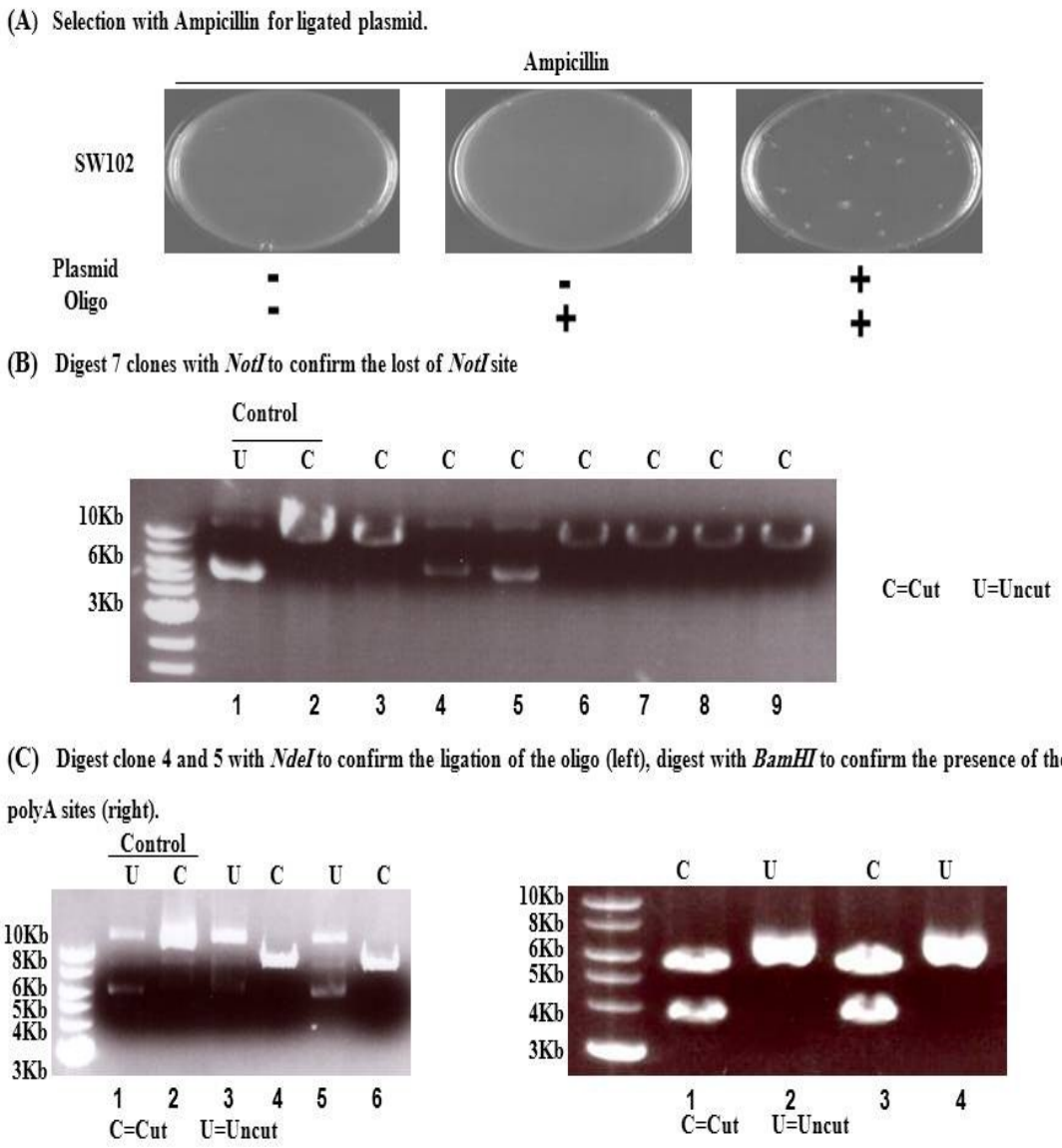


Figure 3.7: The third step in adapting pLSL-TOPO for BAC recombineering. (A) Successful clones carrying the ligated unique sequence (*AvrI - I-SceI – PmeI – I-CeuI – NdeI*) cloned into *NotI* digested pLSL-TOPO-Neo. (B) Two positive clones (clone 4 and 5) showing the loss of the *NotI* unique restriction site after successful ligation. (C, left) Positive clones show the acquisition of an *NdeI* unique restriction site after successful ligation. (C, right) The four SV40 polyA sites are still intact.

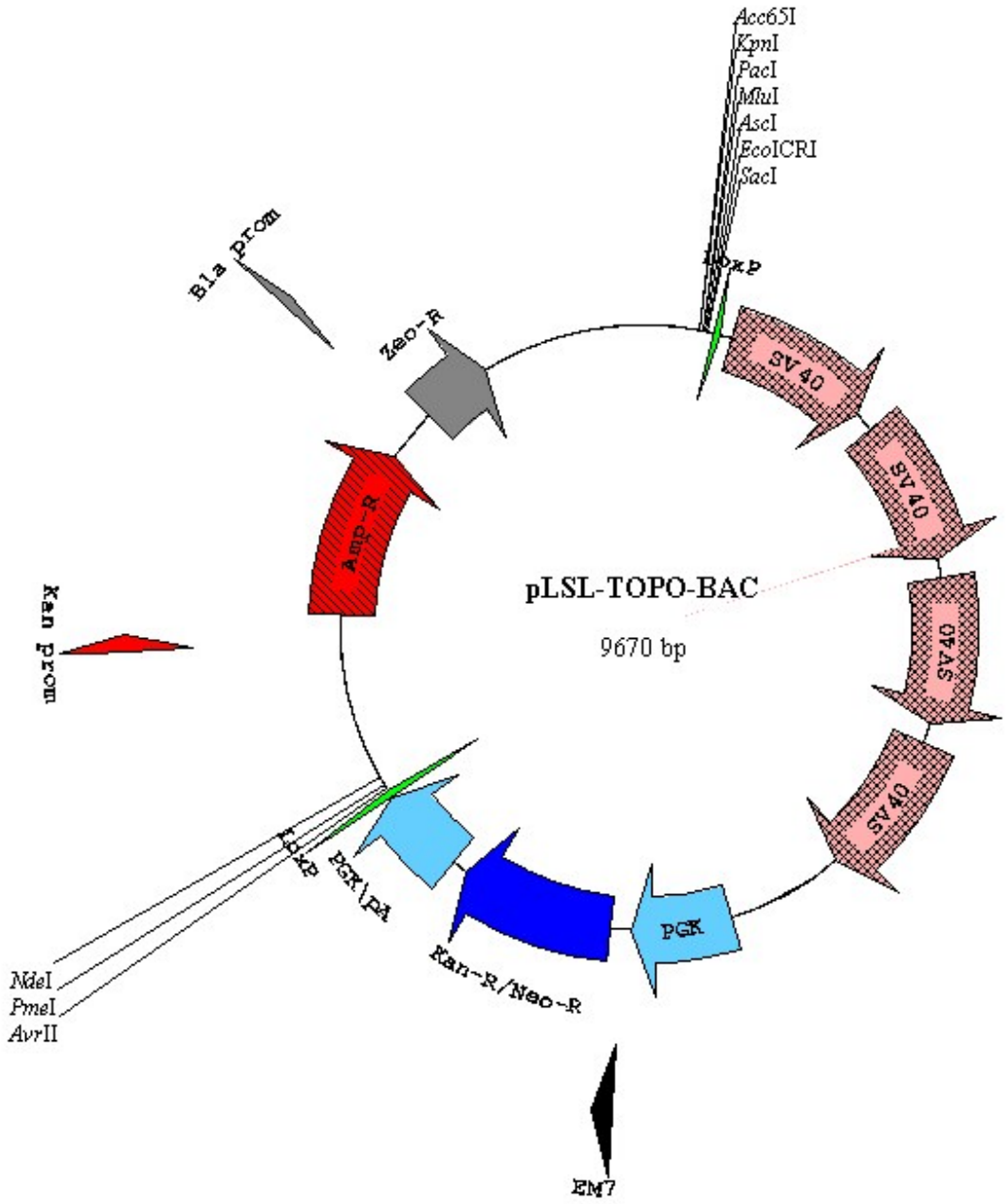


Figure 3.8: pLSL-TOPO-BAC. The new unique sequence (*NdeI*, *PmeI*, and *AvrII*) for homologous arm cloning is shown. The four SV40 polyA sites (pink) are still intact. Figure created using SimVector 4.5.

3.3.4 Cloning 3′ and 5′ β-catenin homologous arms into pLSL-TOPO-BAC unique restriction sites

β-catenin BAC was ordered from Geneservices (bMQ259H05) and BAC DNA was purified; 100ng of BAC DNA was used as a PCR template to amplify 3′ and 5′ homologous arms. The primers used in the PCR reaction were:

5′ recombineering arm

Forward primer(*PacI* site)extra site

5′-CGCGTCGCTTAATTAACGTATGCCTACAATCTGTTT-3′

Reverse primer(*AscI* site)extra site

5′-GCGCATCGTAGGCGCGCCGCCAGTGGTAGTGTGCAG-3′

3′ recombineering arm

Forward primer(*AvrII* site)extra site

5′-CGCGTCGCCCTAGGTTGGTGAAATAATCAGCAAGC-3′

Reverse primer(*NdeI* site)extra site

5′-GCGCATCGTACATATGTGCTGCCAGTGGCTGAC-3′

Each PCR cycle was performed at 95°C for 1.5min (denaturation step) followed by 55°C for 30sec (annealing step) followed by 72°C for 45sec (elongation step) for 40 cycles. The PCR product was purified using a PCR clean-up kit (Qiagen) and run on 2% agarose gel to confirm successful PCR (Figure 3.9.A and 3.10.A).

3.3.4.1 Cloning the 3′ β-catenin homologous arm into pLSL-TOPO-BAC *AvrII* and *NdeI* sites

To clone a 3′ homologous arm, 300ng of pLSL-TOPO-BAC and 5µl of the 3′ homologous arm (PCR product) were double-digested individually with *NdeI* and *AvrII* for 1hr. Three different ratios (1:1, 1:3, and 3:1) of cut-arm and cut-plasmid were ligated using T4 DNA ligase at 16°C for 18hr. The whole ligation mix was digested with *PmeI* for 1hr to minimize self-ligated plasmid transformation. Following digestion, DNA was transformed into DH5α bacteria strain and bacteria were then grown on ampicillin agar plates. Successful colonies were picked and digested again by *PmeI* (Figure 3.9.B). Successful clones were further tested by double digestion using *NdeI* and *AvrII* and successful cleavage of the 3′ arm confirms the integration (Figure 3.9.C).

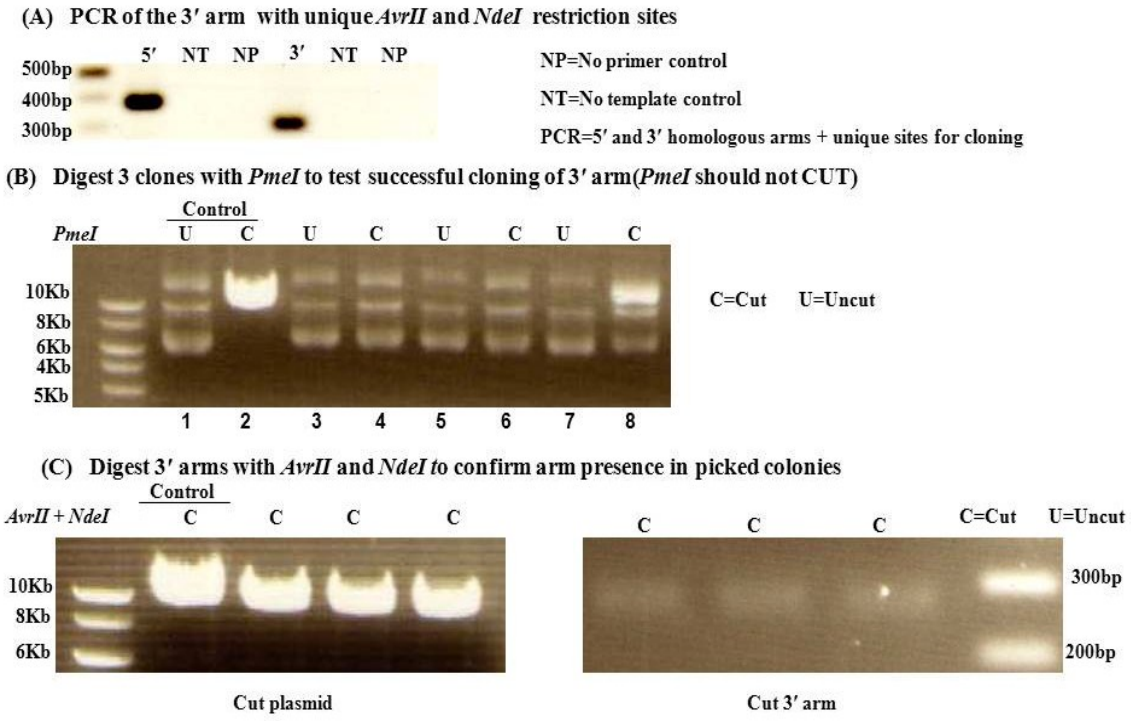


Figure 3.9: Cloning the 3′ homologous arm into pLSL-TOPO-BAC *NdeI* and *AvrII* sites. (A) Successful amplification of the 3′ homologous arm from β-catenin BAC. (B) Positive clones with successful ligation of the 3′ arm have lost the unique *PmeI* restriction site. (C) Positive clones can be double-digested with *AvrII* and *NdeI* to show cut version of the plasmid (left) and to cleavage of the clones 3′ arm (right).

3.3.4.2 Cloning the 5′ β-catenin homologous arm into pLSL-TOPO-BAC *AscI* and *PacI* site

To clone the 5′ homologous arm, 300ng of pLSL-TOPO-BAC and 5µl of the 5′ homologous arm (PCR product) were double-digested individually with *AscI* and *PacI* for 1hr. Three different ratios (1:1, 1:3, and 3:1) of cut-arm and cut-plasmid were ligated using T4 DNA ligase at 16°C for 18hr. The whole ligation mix was digested with *MulI* for 1hr to minimize self-ligated plasmid transformation. Following digestion, DNA was transformed into DH5α bacteria strain, and bacteria were grown on ampicillin agar plates. Successful colonies were picked and digested again by *MulI* (Figure 3.10.B). Successful clones were further tested by double digestion using *AscI* and *PacI* and successful cleavage of the 5′ arm confirms the integration (Figure 3.10.C, centre and left). The presence of four SV40 polyA sites was also checked using *BamHI* digestion (Figure 3.10.C, right). Once 3′ arm and 5′ arms had been successfully cloned into pLSL-TOPO-BAC, the plasmid was sent for sequencing to confirm arm integration, and the final plasmid was named pLSL-TOPO-β-catenin.

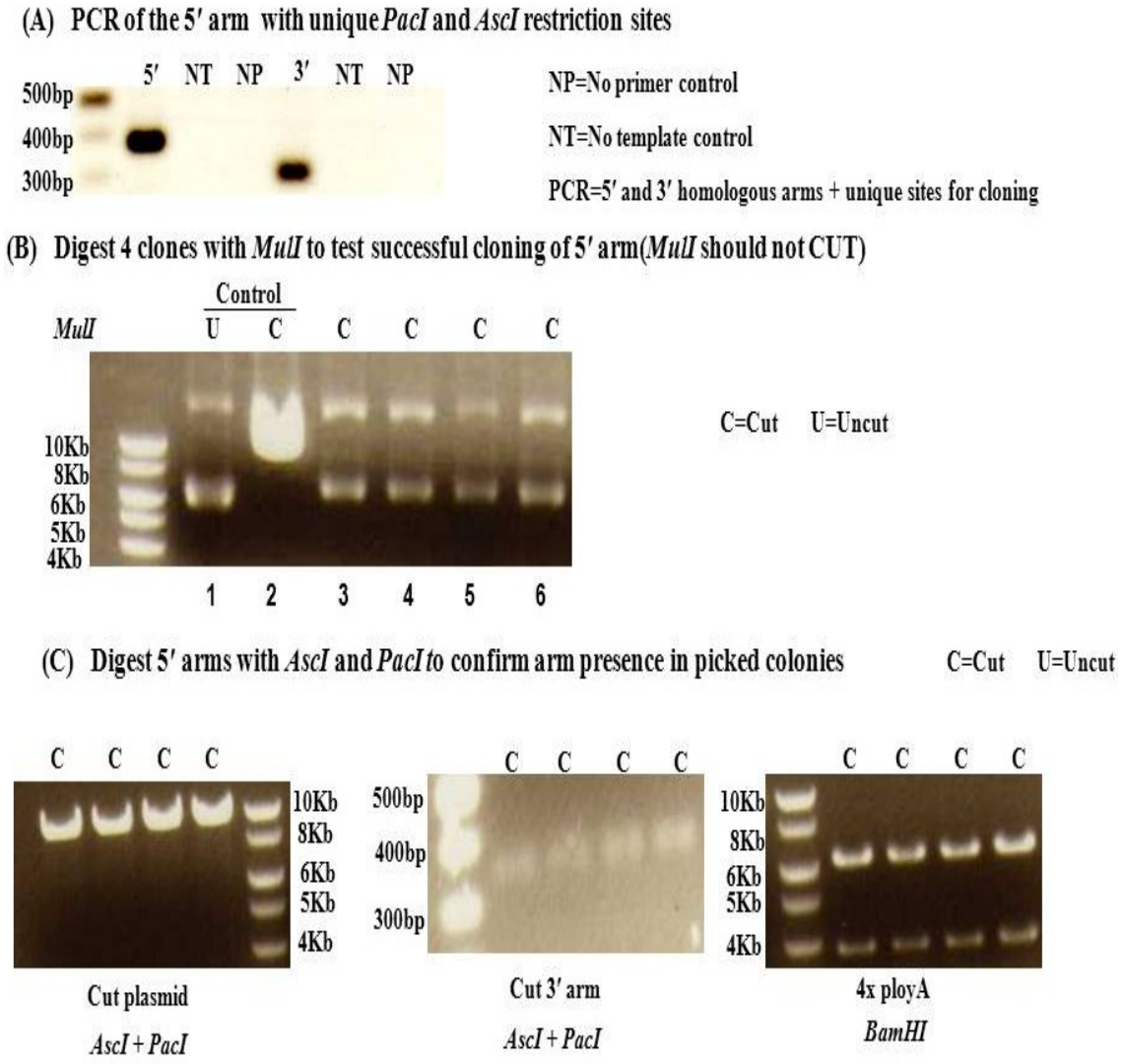


Figure 3.10: Cloning the 5′ homologous arm into pLSL-TOPO-BAC *PacI* and *AscI* sites. (A) Successful amplification of the 5′ homologous arm from β-catenin BAC. (B) Positive clones with successful ligation of the 5′ arm have lost the unique *MulI* restriction site. (C) Positive clones can be double-digested with *PacI* and *AscI* to show cut version of the plasmid (left) and to cleavage of the clones 5′ arm (middle); in addition, *BamHI* digestion (right), confirms the four polyA sites are still intact.

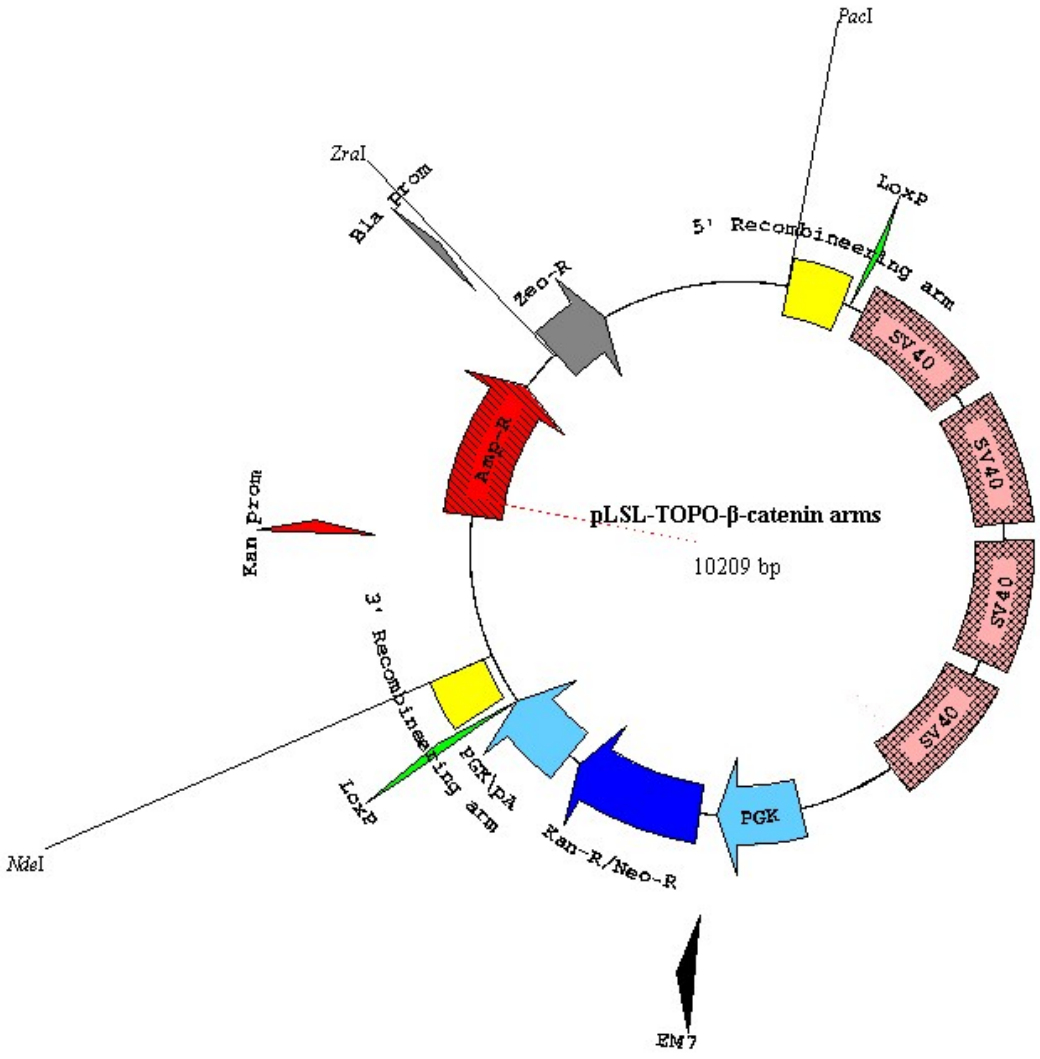


Figure 3.11: pLSL-TOPO-β-catenin arms. Both recombineering 3′ and 5′ arms are shown in yellow. *NdeI* and *PacI* could be used to cleave the BAC targeting cassette. *NdeI* could be used to digest the remaining backbone to minimise false-positive colony formation.

3.3.5 Cloning the LoxP-STOP-LoxP cassette into β-catenin BAC

Once pLSL-TOPO β-catenin was created, it was important to retrieve the β-catenin exon/intron sequence fragment from the BAC. BAC DNA was purified and electroporated into SW102 cells, and the β-catenin BAC profile was checked on agarose gel after *HindIII* digestion. This revealed BAC instability in SW102 (data not shown); thus, the β-catenin BAC was left in original *E.coli* host strain and recombineering proteins (Exo, Beta, and Gam) were brought to the BAC hosting cells using pSim6 plasmid (kindly given by Dr. Peter Hohenstein). The β-catenin BAC profile was checked again to confirm BAC stability following pSim6 electroporation into host strain (data not shown).

A 6kb fragment containing 5′arm-LoxP-STOP-LoxP-EM7-PGK-KanR-NeoR-3′arm (Figure 3.12.A) was extracted from agarose gel after digesting pLSL-TOPO-β-catenin with *NdeI*, *PacI*, and *ZraI* for 2hr, the fragment was re-run on agarose gel after purification to confirm the presence of a clean-single band (Figure 3.12.B). Transforming 1µl of gel purified 6kb fragment into DH5α tested for the presence of any circular plasmid in the DNA; when no growth was observed on agar plates, 1µl of gel purified 6kb fragment was electroporated into the induced β-catenin BAC host strain containing pSim6 to allow recombination, and cells were allowed to recover for 1hr at 32°C without antibiotic. Following the recovery hour, fresh LB containing ampicillin, kanamycin, and chloramphenicol was added to cells, which were grown overnight at 32°C.

Twelve clones were picked and BAC DNA was extracted. Using 50ng of BAC DNA for a PCR template, PCR primers were designed to test the integration of 6kb fragment into β-catenin BAC between exon 2 and 3 (Figure 3.13) by amplifying a product of 550bp that overlap 5′ arm and BAC sequence (upstream of fragment) and a product of 470bp that overlap 3′ arm and BAC sequence (downstream of fragment).

Each PCR cycle was performed at 95°C for 45sec (denaturation step) followed by 56°C for 30sec (annealing step) followed by 72°C for 1min (elongation step) for 40 cycles using the following primers:

5′ recombineering arm check

Forward primer

5′-CATTGCCTCCTGGCTATTGG-3′

Reverse primer

5′-TAAGTCTGCAGGTCGAGGGACC-3′

3′ recombineering arm check

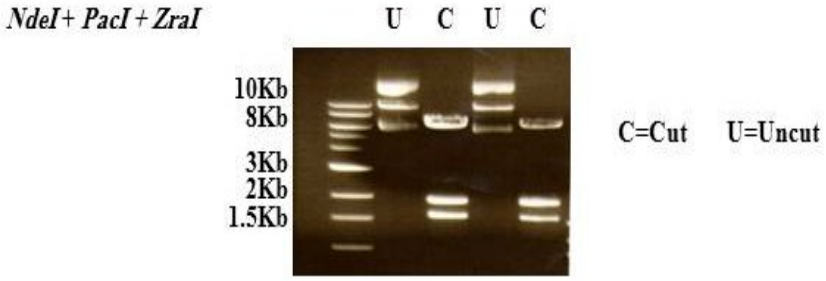
Forward primer

5′-AGCCTCTGTTCCACATACAC-3′

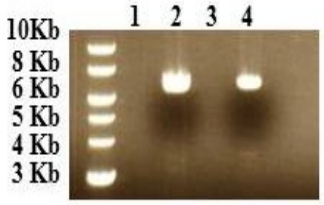
Reverse primer

5′-ATCTTCTTCCTCAGGGTTGC-3′

(A) pLSL-TOPO-β-catenin double digestion with *NdeI* and *PacI*, followed by *ZraI*.



(B) Gel extraction of the 6.5kb fragment created in step (A)



(C) Confirming the integration of LoxP-STOP-LoxP cassette into β-catenin BAC

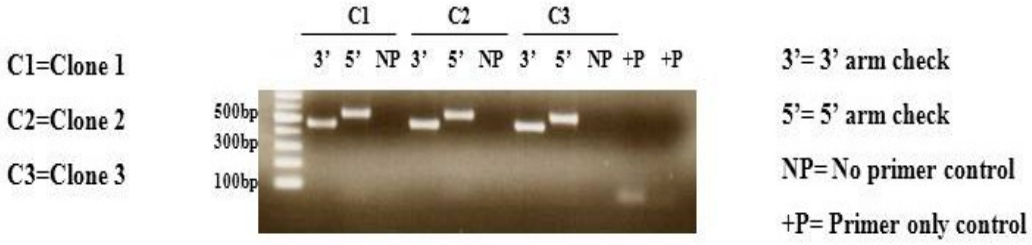


Figure 3.12: Checking the right integration of LoxP-STOP-LoxP cassette into β-catenin BAC. (A) Digestion of pLSL-TOPO-β-catenin with *NdeI*, *PacI*, and *ZraI* revealed a 6.5kb fragment containing the LoxP-STOP-LoxP cassette and the remaining plasmid backbone (1.5kb and 2.0kb). (B) The 6.5kb fragment containing the LoxP-STOP-LoxP cassette after gel extraction and clean-up. (C) Checking correct integration of LoxP-STOP-LoxP cassette into β-catenin BAC by PCR amplification revealed products of the expected size (550bp for the 5′ arm and 470bp for the 3′ arm).

3.4 Discussion

It was important to check the working template of pLSL-TOPO once received from Addgene to make sure that the plasmid was as it should be. A few digestion tests using *NotI* (cut once) and *BamHI* (cut twice) confirmed the presence of the right plasmid (Figure 3.3.A). The main characteristic of pLSL-TOPO is the presence of LoxP-STOP-LoxP (Figure 3.2), and it was important to confirm the presence of these four SV40 polyA sites every time a modification is performed as losing one of these polyA site could affect the ability to control the expression of mutated protein; thus, regulation of expression of the knock-in gene would no longer be conditional. A good test plan was achieved using *BamHI* digestion, which cut upstream and downstream of the LoxP-STOP-LoxP cassette resulting in a band of (3.9kb) – the right size for the expected cassette. Any loss of a polyA site during a modification step would be noted by the appearance of a band less than 3.9kb after *BamHI* digestion.

The ampicillin gene was successfully amplified from pRosa26/*LacZeo* (Figure 3.3.A), where linearizing the template and digesting methylated DNA following PCR amplification helped to reduce the appearance of any false-positive colonies, as the PCR template (pRosa26/*LacZeo*) could cause ampicillin resistance. The issue of plasmid copy number in the host strain could cause some complications, as a single copy of the right plasmid in comparison to multiple copy of the wrong plasmid would still give antibiotic resistance even though the majority of copies of the plasmid in the cell are incorrect. To overcome this issue, re-transformation of the plasmid by electroporation is advisable (Thomason *et al*., 2007) after every step of modification. Successful cloning of the ampicillin gene was confirmed by ampicillin and zeocin resistance (Figure 3.3.B), kanamycin sensitivity (Figure 3.3.C), and *KpnI* single digestion, which resulted in a 9kb band (Figure 3.3.D) – the expected size for the correct plasmid, and not a 14kb band which would indicate the presence of the PCR template. *BamHI* digestion also confirmed the presence of four polyA sites as expected (Figure 3.3.E).

The EM7/Kanamycin sequence was cloned using a similar approach to that for the ampicillin sequence, but a different testing strategy was designed. *PstI* should cut twice in the correct clone: once upstream of the four polyA sites and again in the Kan/Neo-R gene (Figure 3.6). So *PstI* digestion, in one test, checked both the insertion of the Kan/Neo-R fragment and the number of polyA sites. However, a *PstI* digest would result in two almost equal sized fragments, which would not be clearly resolved by agarose gel electrophoresis. However, a double digest using *PstI* and *NotI*, which further digested one of the *PstI* fragments (the one that does not contain the four polyA sites), would resulted in three different-sized fragments [1196bp, 3328bp and 4779bp (Figure 3.5)] that were easily separated on an agarose gel. The 4779bp fragment contains the four polyA sites, so any loss of polyA signal would have been identified by the production of a fragment smaller than 4779bp by 650bp for each polyA site lost.

The 5′ homologous arm was slotted easily between the *PacI* and *AscI* sites upstream of the LoxP-STOP-LoxP cassette. However, the 3′ homologous arm needed to be slotted into a unique restriction site downstream of the LoxP-STOP-LoxP cassette, and such a site did not exist in the original plasmid. Thus, a unique DNA sequence was designed in a way that allowed the insertion of the following restriction endonuclease sites into the *NotI* site: *AvrII – IsceI – PmeI – IceuI – NdeI*. The oligos were designed so that the *NotI* site was abolished after successful ligation of the insert sequence. All these five sites are then unique in the plasmid. A simple conventional cloning step then involved digesting pLSL-TOPO-Neo with *NotI* followed by sequence ligation; however, a further *NotI* digestion after ligation and before transfection assisted in minimising false-positive colony formation (Figure 3.8). A final digestion test using *NotI*, *NdeI*, and *BamHI* confirmed the loss of the *NotI* site in two clones, clone 4 and 5 (Figure 3.7.B), acquisition of the new unique sites (Figure 3.7.C, left), and the presence of four polyA sites (Figure 3.7.C, right).

At this stage the pLSL-TOPO-BAC is a universal BAC targeting vector which can be used to target any BAC of interest. However, it is important at this stage to pick a gene and a desired subtle mutation(s) as the following steps are gene-dependent. It is very important when using this approach to have the LoxP-STOP-LoxP cassette toward the start of the gene (5′ end) to eliminate any dominant negative effects of any stable partial-protein expression (up to the stop codon); in addition, it is also important to keep the distance between the LoxP-STOP-LoxP cassette and the actual mutation as small as possible since the selection during the embryonic stem cell targeting stage is done on the LoxP-STOP-LoxP cassette: the bigger the distance between cassette and mutation, the bigger the risk of picking up a clone that has the stop cassette but not the desired mutation (owing to a sliding holiday junction between the genomic DNA and the targeting construct resolving by chance before the actual mutation). After careful consideration of gene structure (exon/intron) and desired mutations, mouse β-catenin (Ctnna1 ENSMUSG00000037815) with the desired (S33Y) and (S45Δ) mutations were chosen, as β-catenin and its desired mutation had the best genomic structure for this type of BAC targeting approach (Figure 3.13, B).

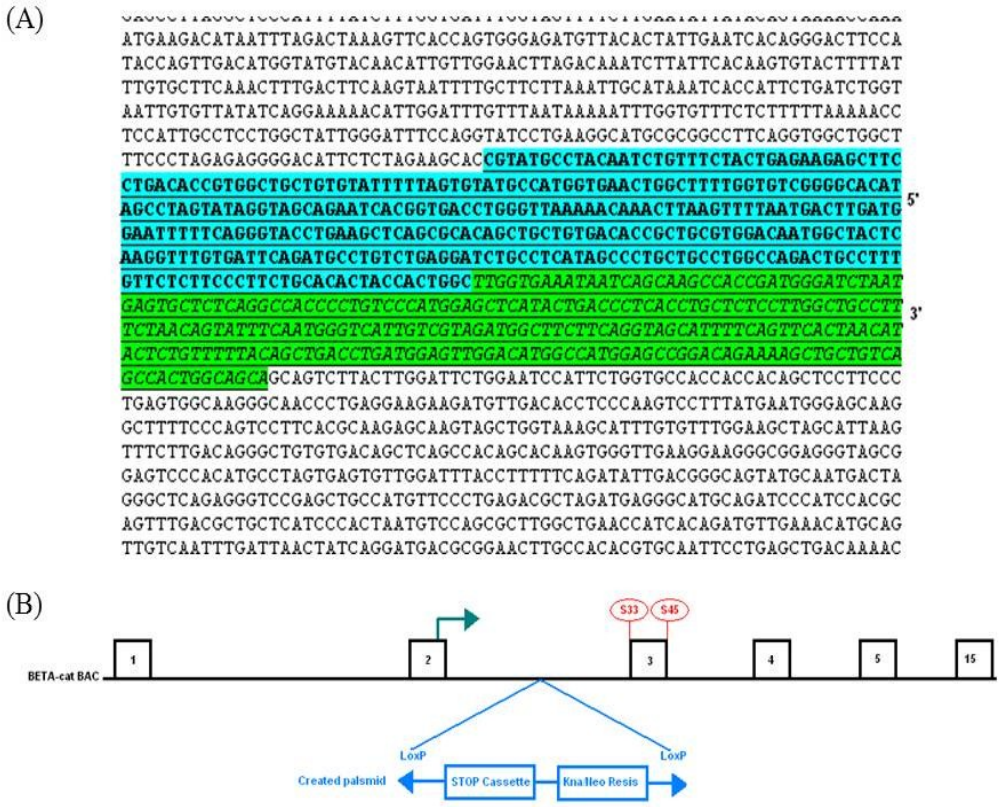


Figure 3.13: β-catenin BAC DNA sequence. (A) The sequence of 5′ homologous retrieval arm (blue) and 3′ homologous retrieval arm (green). (B) A diagram showing the exact area between exon 2 and exon 3 where LoxP-STOP-LoxP cassette to be targeted, the location of desired mutation(s), and the distance between the 5′ end of β-catenin and the stop cassette.

An important consideration in 5′ and 3′ homologous arm primer design was the enzyme docking site, as most restriction endonucleases dock a few base pairs downstream of the actual cutting site so 8–10 random base pairs were attached to the PCR sequence to act as the enzyme docking site. It was also important to choose three unique restriction enzymes during arm cloning, where the arm is cloned between the first and the third site, and the second site is used to cut any undigested plasmid to minimise false-positive colony formation. These considerations increase the efficiency of homologous arm cloning into pLSL-TOPO-BAC vector.

The 3′ arm (258bp) was amplified using the β-catenin BAC (Figure 3.9.A) and cloned between *AvrII* and *NdeI*, where *PmeI* (Figure 3.9.B) was used to cut any self-ligated plasmid. Once cloned, plasmid was digested once one using *AvrII* and *PmeI* to confirm arm cleavage and plasmid digestion (Figure 3.9.C). The 5′ arm (344bp) was amplified using the β-catenin BAC (Figure 3.10.A) and cloned between *AscI* and *PacI*, where *MulI* (Figure 3.10.B) was used to cut any self-ligated plasmid. Once cloned, plasmid was digested once one using *AscI* and *PacI* to confirm arm cleavage and plasmid digestion (Figure 3.10.C, left and middle). In addition, *BamHI* confirmed the presence of four polyA sites (Figure 3.10.C, right).

Once homologous arms were cloned into the BAC targeting vector (Figure 3.11), it was important to cleave the stop cassette AND digest the remaining backbone (in an individual reaction) using *ZraI* or any another suitable enzyme, as this should cut any circular plasmid where the stop cassette hasn't been cleaved because these plasmids could give false-positive colonies from the kanamycin resistance gene located on the backbone (Figure 3.12, A). In addition to this, it is advised to test the quality of the stop cassette extracted from the gel (Figure 3.12, B) by transforming a small amount into Dh5α cells to test for the presence of any circular plasmid in the gel fragment. All these considerations help to increase the efficiency of cloning the stop cassette into the desired BAC.

A correct integration could be confirmed by designing PCR primers that overlap an area of a few hundred base pairs between the BAC sequence and the homologous arm in both locations (3′ and 5′) and amplification of a product would confirm the integration of the stop cassette at the right location (Figure 3.12, C).

At this stage, it has been confirmed that the LoxP-STOP-LoxP cassette has been targeted into β-catenin at the exact area between exon 2 and 3. BAC DNA containing the cassette was still present in the original BAC hosting strain where recombineering proteins are expressed by pSim6 plasmid (ampicillin resistance). Many attempts were made to create the desired S33Y and S45Δ mutation on the BAC sequence; however, all these attempts were unsuccessful but will be mentioned briefly (without showing the data) for discussion purposes.

The first approach attempted is described by Yang *et al* (2003). This technique is designed for low copy, large plasmid manipulation such as BACs and involves a simple two-step method, named Hit-and-Fix, to generate subtle mutations in BACs. The method involves electroporating the denatured PCR fragment that contain 20bp of unique sequence (including an *XhoI* site) flanked by 80bp of sequence homologous to the area that needs to be targeted: after transfection, clones are tested by PCR for the unique sequence, followed by *XhoI* confirmation (one big limitation). Once the sequence is hit, it gets fixed by transfecting the original 20bp (containing the desired mutation) flanked by the same homologous arms used in the hit. We tried trouble shooting a few times before deciding to move on to the second approach.

The second approach attempted is described by Warming *et al* (2005), and this approach is also designed for low copy, large plasmids such BACs. The technique involves hitting the desired sequence for mutation by *GalK* (galactokinase) flanked by homologous arms; this gene will allow the host strain to grow on minimal medium containing galactose as the only carbon source.

Once clones with *GalK* are selected, the *GalK* gene could be replaced using the same homologous arms with the desired mutation followed by growing cells on minimal medium, glycerol (carbon source), and DOG (2-deoxy-galactose) where cells with the *GalK* gene (but not carrying the desired mutation) will catalyse the phosphorylation of DOG leading to a toxic build-up of 2-deoxy-galactose-1-phosphate (Alper and Ames 1975). However, it is important to use a strain which is *GalK* negative such as SW102 to allow *GalK* selection. No successful clones were achieved from this approach either.

At this stage I had problems with BAC DNA purification and BAC stability; we decided to clone the desired β-catenin sequence from BAC into the final stem cell targeting vector (pL611-kindly given by Peter Hohenstein) then create the desired mutation on the plasmid. At this stage we were faced with two choices: to clone the BAC β-catenin sequence containing the LoxP-STOP-LoxP cassette into pL611 resulting in a plasmid with an excess size of 20kb, or to clone the BAC β-catenin sequence without the stop cassette (expected size 14kb) followed by cloning the stop cassette once mutation is created. We decided to clone the β-catenin sequence into pL611 without the stop cassette to deal with 14kb plasmid instead of 20kb as this should increase the chances of creating the desired mutation.

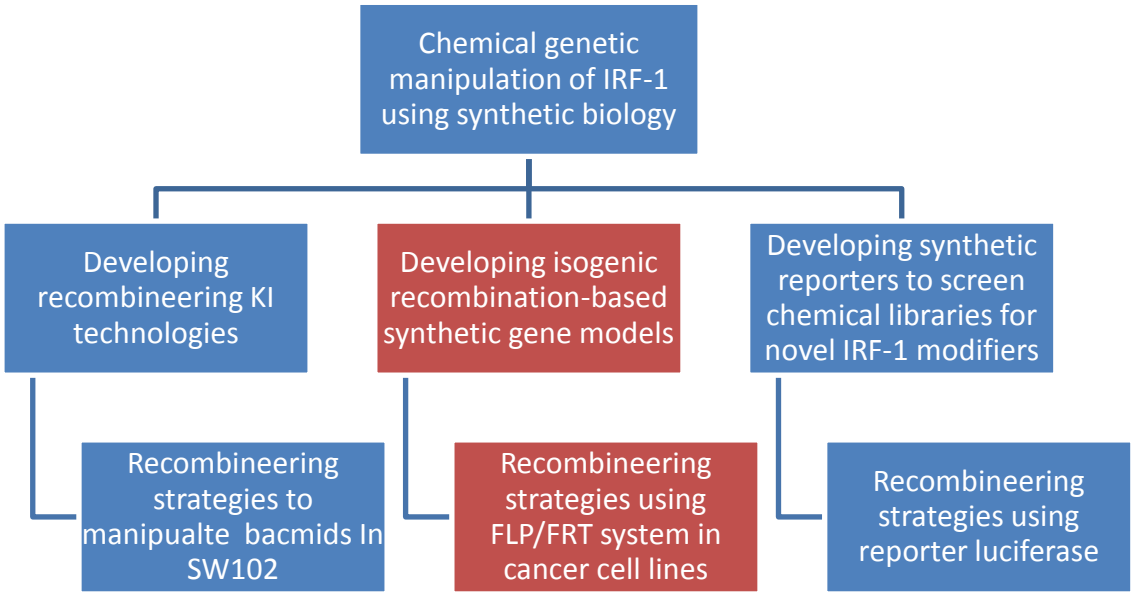
PCR primers containing sequence homologous to the BAC DNA that need to be cleaved was used to amplify pL611, once amplified, pL611 was electroporated into BAC host cells with pSim18 (another recombineering protein vector, kindly given by Peter Hohesntein). Clones with a successful recombineering were picked and re-transformed into DH5α cells, plasmid DNA was purified and checked by digestion. At this stage, we successfully had cloned the desired 10kb fragment from β-catenin BAC into pL611, making a plasmid with a total size of 14.7kb (data not shown), and the plasmid was named (PL611- β-catenin). To test the possibility of cloning the LoxP-STOP-LoxP cassette after a mutation is created, we decided to test the efficiency of such a cloning procedure before progressing with the mutation. We successfully cloned the stop cassette into PL611- β-catenin, as shown before resulting in a plasmid with an excess size of 20.8kb (data not shown) plasmid was named PL611- β-catenin-STOP.

We repeated the *GalK* method and the Hit-&-Fix method on both PL611- β-catenin and PL611- β-catenin-STOP plasmids without any successful results, so we also attempted new approach described by Wang *et al* (2009) where sequence is first hit by a recombineering cassette containing kanamycin resistance and bacterial ribosomal protein *rpsL*$^{+}$ gene. Once the cassette has integrated at the site, clones could be selected using kanamycin resistance followed by a second recombineering hit using the same homologous arms to knock out the cassette and knock in the mutation so cells could be selected on streptomycin plates, as *rpsL*$^{+}$ confers streptomycin sensitivity (Stavropoulos and Strathdee 2001). As this approach involves kanamycin resistance in the first step it was only possible to perform it on PL611- β-catenin and β-catenin BAC. However, no positive clones with the desired mutation were obtained. In addition, we also attempted the GENRART® Site-Directed Mutagenesis System for plasmid up to 14kb (Invitrogen) on PL611- β-catenin (14.7kb); however, all colonies tested were carrying multiple unwanted mutations. Finally, we tried one last approach – the In-Fusion® PCR Cloning system from Clonetech; however, no positive clones were detected.

Chapter 4: Developing an isogenic recombination-based synthetic gene model

4.1 Chapter abstract

The Flp-In System™ from Invitrogen eased the generation of isogenic stable mammalian expression cell lines by adapting the DNA recombination system in *Saccharomyces cerevisiae*. Using this system, two isogenic stable cell lines were created: one overexpresses wild-type IRF-1 and the second overexpresses a mutant that abolishes IRF-1 DNA binding ability (W11R). Both cell lines were investigated, using microarray analysis, to identify the panel of IRF-1 downstream targets. Twelve potential targets were validated using qRT-PCR to confirm the increase in their expression level once IRF-1 is overexpressed. In addition, *in-silico* analysis of their putative promoter regions was undertaken to identify the presence of any common DNA binding motifs such as ISREs.



4.2 Chapter introduction

Genetically modified stable cell lines are standard tools in cancer studies; they are considered to be of more physiological relevance to the human body, compared to traditional transient transfection, making them a great tool in the cancer field. However, the random integration of a gene of interest during stable cell line creation is a big limitation in creating stable cell lines using the traditional method, especially when isogenic cell lines are required. There is a need for a modified method for creating stable cell lines where any gene of interest could be slotted in at a specific genomic location in the host cell line. Such a method could contribute toward cancer therapy and understanding the mechanism of cancer.

4.2.1 The Flp-In System™

The Flp-In System™ from Invitrogen introduced a new system for creating stable cell lines that allows the integration and the expression of any gene of interest in any mammalian host cells at a very specific genomic location called FRT site: a 34bp DNA sequence with two 13bp inverted repeats and an 8bp spacer. The system is adapted from the *Saccharomyces cerevisiae*-derived DNA recombination system that uses a recombinase (Flp) and site-specific recombination (Sauer 1994).

There are three important steps in creating stable cell lines using this approach: the first step involves adapting the host stable cell line for FRT integration, and this is achieved by pFRT/*lac*Zeo transfection into the chosen host cell line. Such transfection will allow the random genomic integration of *lacZ*–Zeocin (Figure 4.1) fusion gene, whose expression is controlled by the SV40 promoter, into the host cell line resulting in clones being zeocin resistant. The *lacZ*–Zeocin fusion gene contains an FRT site which has been inserted just downstream of the ATG initiation codon of the fusion gene (Figure 4.1). The second step involves cloning the gene of interest into an FRT expression shuttle vector such as pEF5/FRT/V5-DEST (Figure 4.2) under the control of human EF-1α promoter. Such cloning can be achieved easily using a standard LR reaction between a donor vector carrying the gene of interest and the pEF5/FRT/V5-DEST vector.

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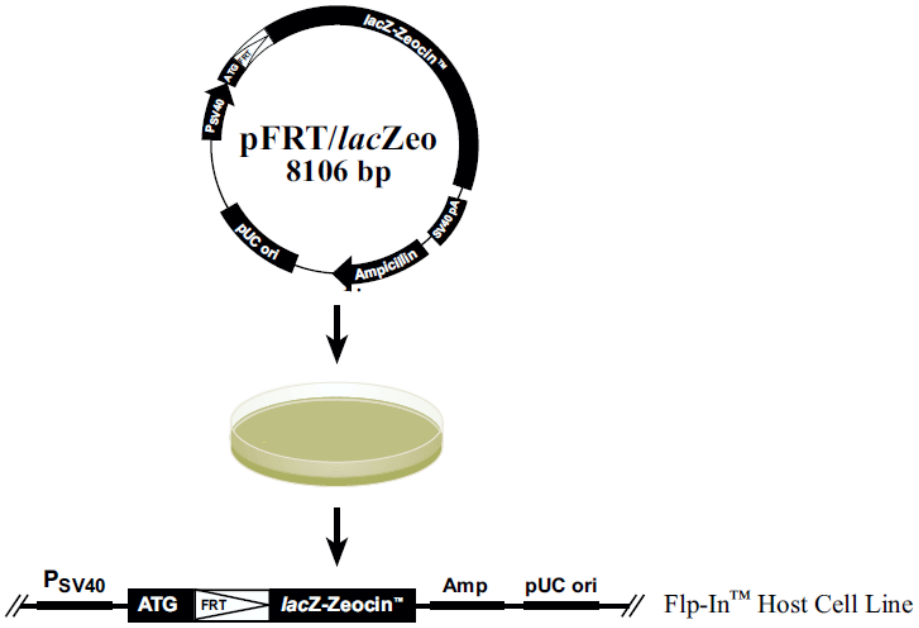


Figure 4.1: Step one in creating FRT mammalian host cell line. pFRT/*LacZeo* is characterized by the *lacZ*–Zeocin fusion gene, whose expression is controlled by SV40 promoter, and the FRT site which has been inserted just downstream of the ATG initiation codon of the fusion gene (Adapted from Flp-In System, Invitrogen).

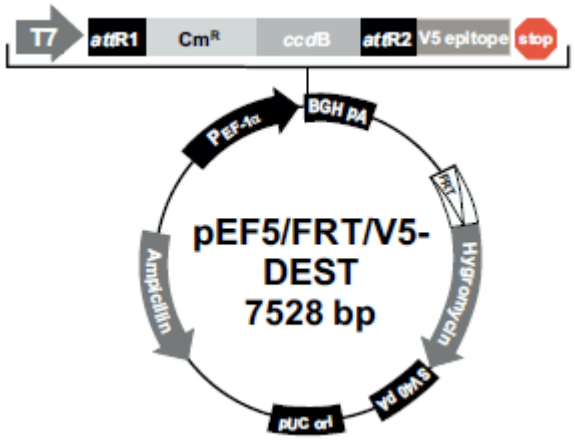


Figure 4.2: pEF5/FRT/V5-DEST. Straightforward cloning of any gene of interest from any Gateway™ Donor vector is facilitated by attR1 and attR2 DNA sites where the resultant flipped gene will be under the regulatory control of the human EF-1α promoter (Adapted from pEF5/FRT/V5-DEST Gateway™ Vector, Invitrogen).

Once a gene of interest is cloned into the FRT expression vector, this vector and a third vector named pOG44 are co-transfected into a host cell line containing the FRT site. pOG44 will then express Flp-recombinase, which will mediate DNA recombination (O'Gorman *et al.,* 1991) between the gene of interest on the expression vector and the FRT site in the genomic DNA (Figure 4.3). As a result of a successful recombination the SV40 promoter and the ATG initiation codon are brought into proximity and frame with the hygromycin resistance gene, resulting in inactivation of *lacZ*–Zeocin fusion gene (Figure 4.3). Thus, clones can now be selected for hygromycin resistance and zeocin sensitivity.

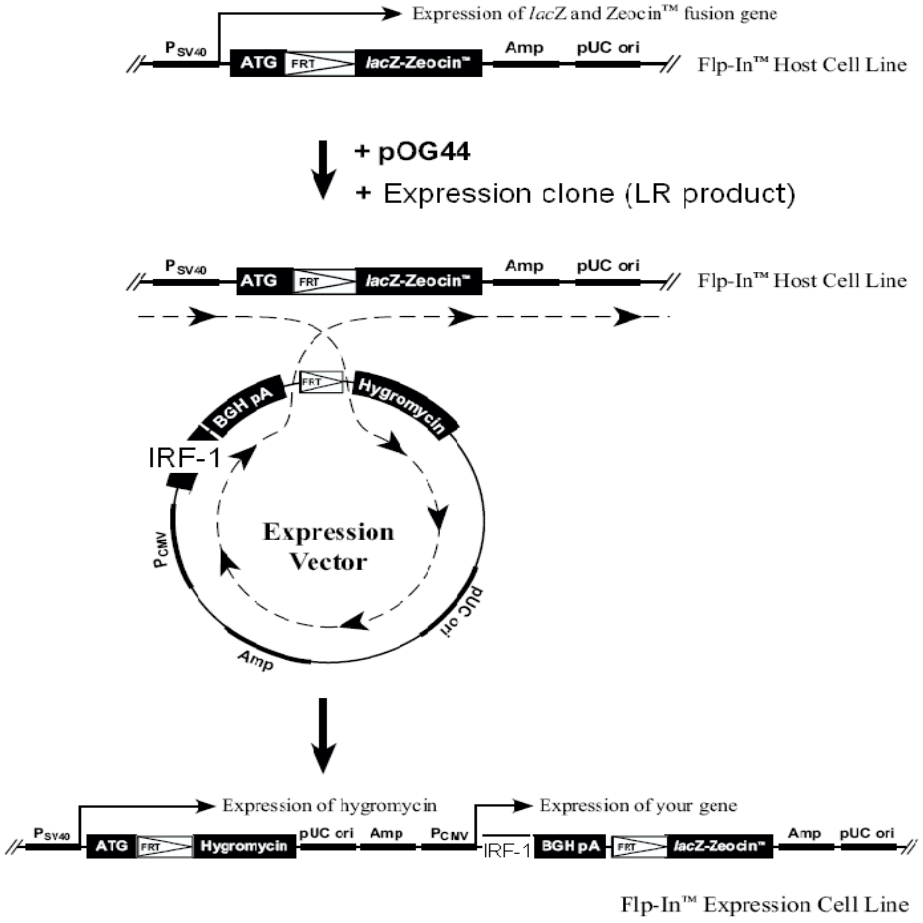


Figure 4.3: Flp-In System™. Illustrative diagram showing the recombineering cloning of a gene of interest from an expression vector into a host cell line with a FRT binding site (Adapted from Flp-In System, Invitrogen).

4.2.2 Interferon Regulatory Factor 1

IRF-1 is a nuclear transcription factor first described in mice as a transcription factor that binds to the upstream regulatory *cis* element region positive regulatory domain I (PRDI), 5′–GAGAAGTGAAAGT–3′, of interferon-β gene following a viral infection (Miyamoto *et al.,* 1988). The same group showed that IRF-1 is also involved in the regulation of different genes such as interferon-α and genes in the major histocompatibility complex (MHC) mainly class 1 (Miyamoto *et al.,* 1988).

As a DNA binding protein, the IRF-1 DNA binding domain has been determined and shown to include three α-helices (the third α-helix is characterized by a GAAA sequence major groove), four anti-parallel β-sheets and three loops thought to be similar to the HTH binding domain but reported to interact by a different mode (Escalante *et al.,* 1998). In all members of human interferon regulatory factors a DNA binding domain characterized by a winged type helix-loop-helix motif, with five tryptophan repeats located near the N-terminal, is present (Uegaki *et al*., 1995). By disturbing these five tryptophan repeats, and mutating tryptophan 11 into arginine, an IRF-1 mutant that abolishes DNA binding ability is created (Eason *et al*., 1999).

Further studies showed that IRF-1 binds to a DNA consensus motif, termed interferon regulatory factor element (IRF-E), characterized by the following sequence: 5′–G (A) $AAA^{G/C\ T/C}GAAA^{G/C\ T/C}$–3′ (Tanaka *et al*., 1993). The IRF-E motif overlaps with another IRF-1 binding motif named ISRE, characterized by a 5′–$^{A/G}$NGAAANNGAAACT–3′ consensus (Darnell *et al*., 1994). The IRF-1 induced genes vary in function from antiviral/antibacterial response genes such as *IFN-β, IFN-α* (Harada *et al*., 1990; Matsuyama *et al*., 1993), Guanylate-binding protein *GBP* (Kimura *et al*., 1994)*, iNOS* (Kamijo *et al*., 1994)*, gp91^{phox}* (Eklund *et al*., 1998) and *OAS2* (Harada *et al*., 1998) to anti-proliferative response genes such as protein kinase R (Samuel *et al*., 1997) and *p21$^{WAF/CIP1}$* (Nguyen *et al*., 1997). In addition, IRF-1 can also induce some apoptosis genes such as *Caspase 1* and *Caspase 7* (Tamura *et al*., 1995; Taniguchi *et al*., 2001).

IRF-1 is also involved in triggering the expression of genes involved in cell transformation inhibition such as lysyl oxidase (Tan *et al*., 1996), T_H1 type immune response such as interleukin-12 (Taki *et al*., 1997), inflammation, natural killer cell differentiation, such as interleukin-15 (Ogasawarak *et al*., 1998), and major histocompatibility complex class I (MHC) expression such as Class II transactivator (Hobart *et al*., 1997). These MHC, mainly class I, could enhance the formation of surface antigens in tumour cells that can be recognized by host defence/immune mechanisms and helping to eliminate tumour cells (De Andrea *et al*., 2002). In addition interferon-γ, with the aid of interleukin-2, can enhance T-cell activation leading to the expression of more major histocompatibility complex class II, in same response described earlier (De Andrea *et al*., 2002). Thus, interferon-based therapy for cancer has been introduced, with other current therapies such as chemotherapy/radiotherapy, to minimize the overall dose of therapy, aiming at a more effective outcome.

However, the complete panel of IRF-1 downstream targets has not been fully characterized; thus, this chapter aimed to analyse IRF-1 targets using microarray technology. To achieve this, stable cell lines over-expressing wild-type IRF-1 needed to be created, as stable cell lines are more physiological relevant compared to transient transfections. To eliminate the possibility of activating genes indirectly through protein–protein interaction, an isogenic stable cell line overexpressing an IRF-1 mutant (W11R) that abolishes DNA binding ability must be created in addition to the wild-type IRF-1. Once isogenic stable cell lines are created using the Flp-In System™ (Invitrogen), potential targets that are overexpressed in wild-type IRF-1, but not in W11R IRF-1, can be identified using microarray analysis. However, microarray analyses need to be validated using qRT-PCR to confirm the change in protein level expression. Successful candidates from qRT-PCR analysis are further examined *in-silico* at their promoter level to identify any common DNA binding motif such as ISRE or IRF-E.

4.3 Methods and results

4.3.1 Creating a stable A375 Flp-In™ host cell line carrying an FRT site

Human amelanotic melanoma cell line (A375) was chosen to be the host of the FRT site integration. 500ng of pFRT/*LacZeo* was digested using *ScaI,* then 100ng of digested plasmid was transfected into wild-type A375 using Attractene (Qiagen) and cells were allowed to recover for 24hr. Following the recovery period, cells were challenged with zeocin for two weeks.

Twelve zeocin resistance clones were picked and expanded into 6cm tissue culture plates for Southern blot analysis. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen), and 1µg of genomic DNA was digested with 100units of *HindIII* for 4hr. Digested genomic DNA was run on 0.8% agarose gel at 10V for 18hr to allow slow separation. Once separated, genomic DNA was immobilized onto Biodyne® nylon membranes (chapter 2). A PCR probe, recognizing 400bp on the *lacZ*-Zeocin fusion gene, was radiolabelled with [α-^{32}P] dCTP and [α-^{32}P] dATP using Prime-It® II Random Primer Labelling Kit (Agilent Technologies) and hybridized onto immobilized genomic DNA for probe detection (Figure 4.4).

It was important to ensure IRF-1 pathway integrity after the random integration of the FRT site into the A375 genome, thus three FRT clones (FRT7, FRT12, and FRT14) were tested for FRT integration effects on the IRF-1 pathway. Tested clones were treated with 25µg/ml poly (I:C) synthetic RNA for 6hr to induce IRF-1 pathway (Figure 4.5).

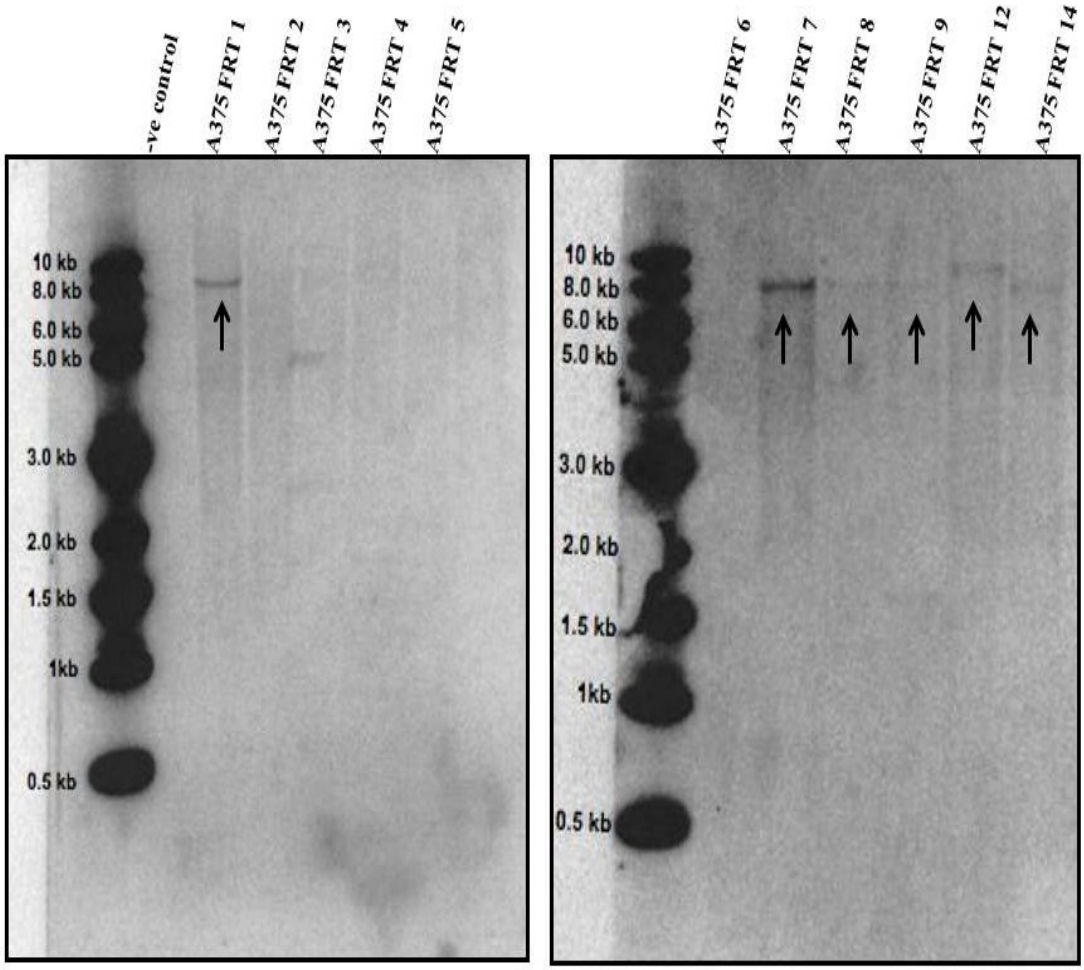


Figure 4.4: Identification of the A375 cell lines with a single FRT site integrated in the genome using Southern blotting. DNA of 12 clones was extracted, digested with *HindIII* followed by Southern blot analysis using a ^{32}P radioactive labelled probe specified for a 400bp region on *lacZ*-Zeocin fusion gene. Results show six potential clones with single FRT site integration (black arrows).

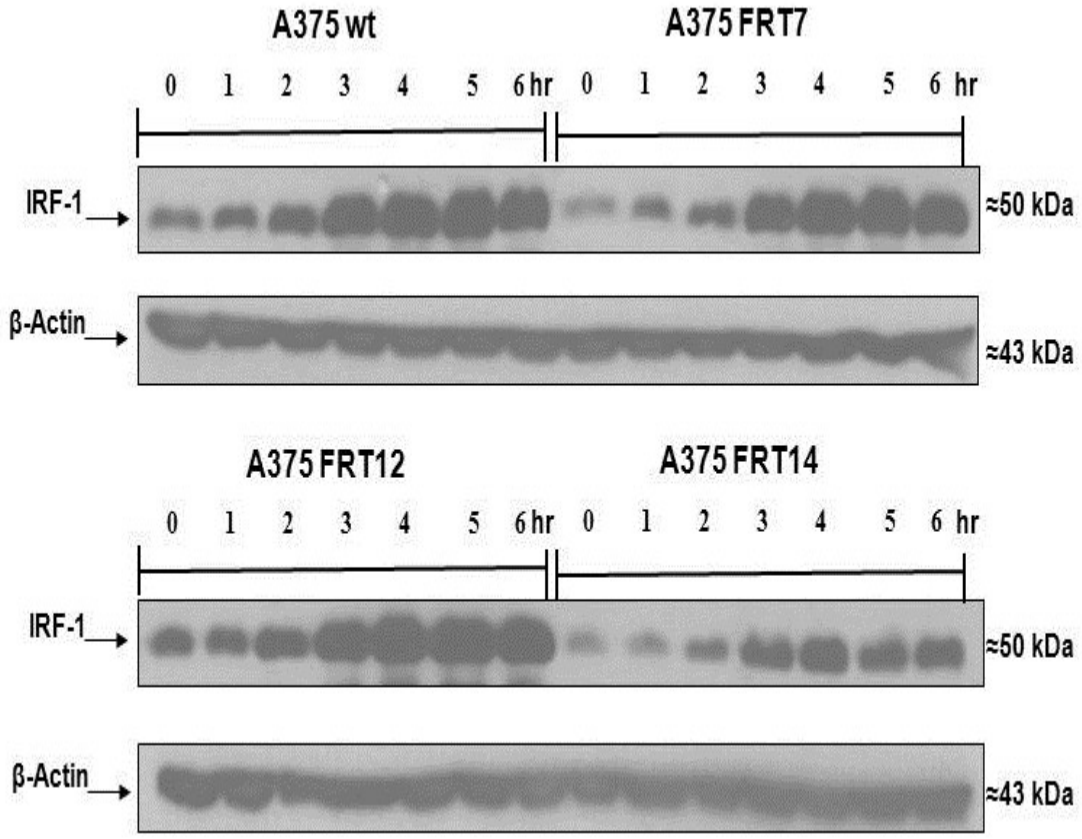


Figure 4.5: Integration of an FRT site does not influence the IRF-1 pathway. Three clones with single FRT integrations were seeded on 6cm culture plates; 24hr later, cells were subjected to a 6hr time course of treatment with 25µg/ml poly (I:C). After harvesting, cells were lysed and subjected to immunoblotting; IRF-1 and β-actin were detected by their respective antibodies. All 3 clones with single FRT site integrations show identical IRF-1 expression level profiles as wild-type A375.

4.3.2 Creating stable A375 over-expressing wild-type IRF-1 and W11R IRF-1

Once the FRT host cell line was created, any gene of interest could be slotted in at the FRT sites to create stable expression cell lines. Two stable cell lines were created, one overexpressing wild-type IRF-1 and the second overexpressing an IRF-1 mutant (W11R) that abolishes IRF-1 DNA binding ability. An LR reaction was performed between pDonr221 carrying wild-type IRF-1 (kindly given by Dr. Vikram Naryan) and pEFF5/FRT/V5–DEST to create pKhal-1, which was sequenced using T7 standard primer to confirm the successful cloning of IRF-1.

The IRF-1 (W11R) mutant was created using site-direct mutagenesis using the following primers:

Forward: 5′–CGGATGCGCATGAGACCCAGGCTAGAGAGATGC–3′

Reverse: 5′–GCCTACGCGTACTCTGGGTCCGATCTCTACG–3′

pKhal-1 (PCR template) was diluted 1:500 and 1µl was used in the PCR reaction. Each PCR cycle was performed at 95°C for 50sec (denaturation step) followed by 55°C for 1min (annealing step) followed by 68°C for 18min (elongation step) for 15 cycles followed by a final elongation step at 68°C for 30min. Once amplified, the DNA was digested with *DpnI* for 2hr to cleave the DNA methylated sites (PCR template). *DpnI*-digested PCR product was transformed into DH5α and grown on ampicillin agar plates overnight at 37°C. Plasmid DNA was extracted, profile digested (data not shown), and potential clones were sequenced using standard T7 primer to confirm the desired mutation. Positive clones with the W11R mutation were named pKhal-2.

A375 FRT7 and A375 FRT14 were seeded onto 10cm tissue culture plates to reach 60-80% confluence the following day, 5µg and 10µg of total DNA pKhal1 or pKhal-2 and pOG44 (1:9 ratio respectively) was transfected using Attractene (Qigen). Transfected cells were left overnight at 32°C without antibiotics; the following day cells were transferred into a 37°C incubator and challenged with 200µg/ml hygromycin for two weeks or until complete death of no DNA control. Surviving clones were mixed together by trypsinzing the whole plates and re-seeding them onto fresh 6cm plates without antibiotic. Cells were tested for their IRF-1 overexpression level using immunoblotting and luciferase assays (Figure 4.6).

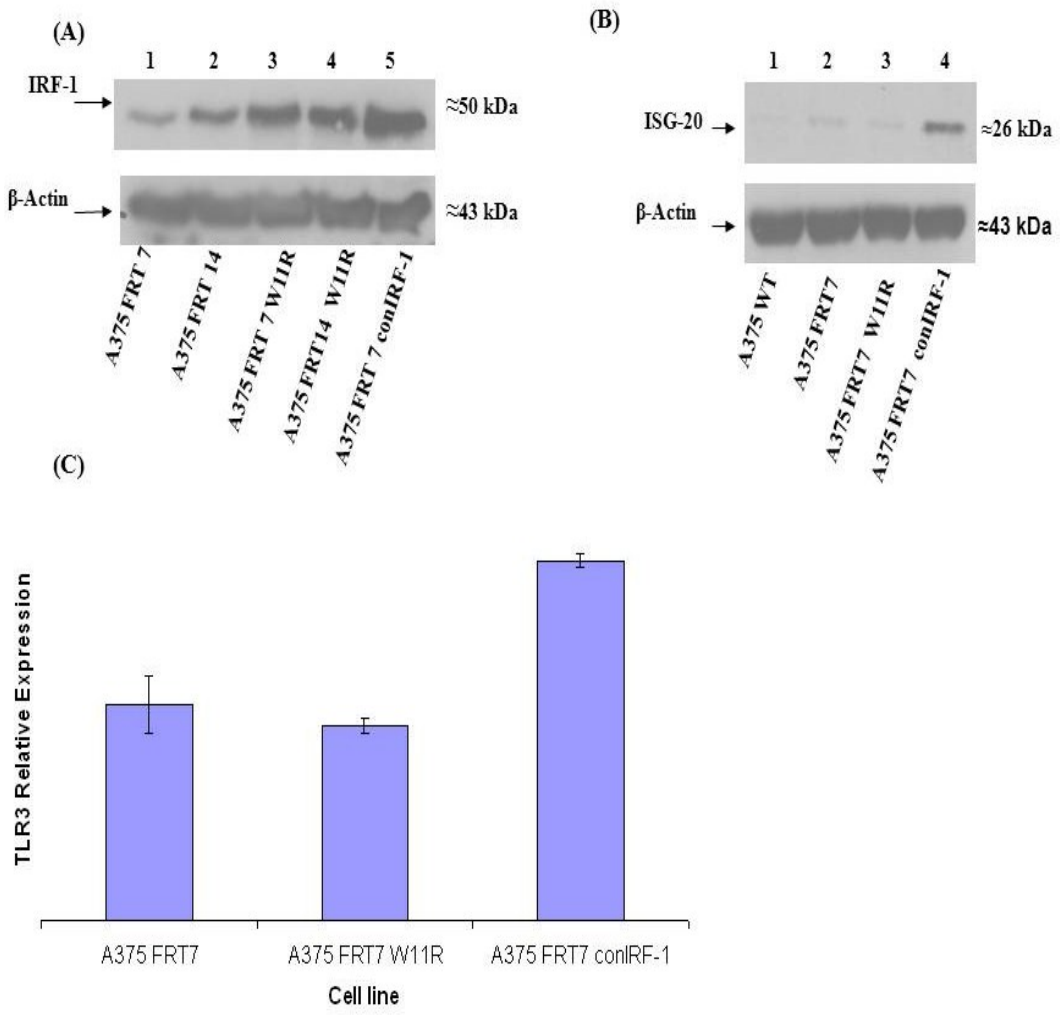


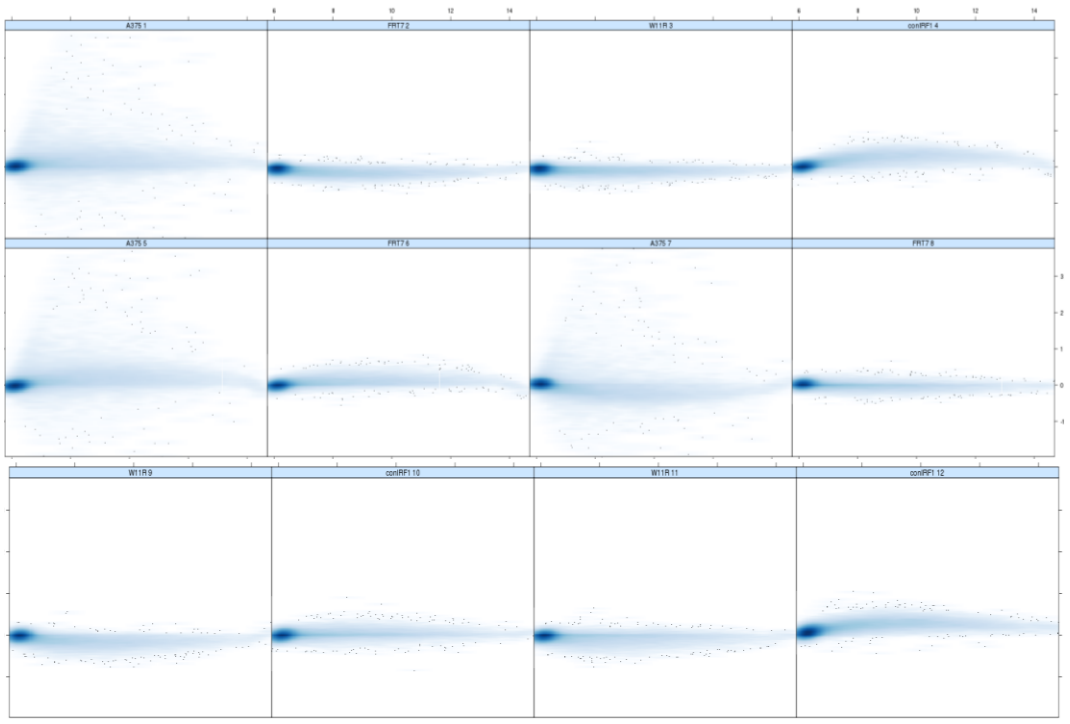
Figure 4.6: The successful creation of wild-type IRF-1 and W11R IRF-1 stable expression cell lines. pKhal-1 or pKhal-2 and pOG44 were co-transfected into A375 FRT7 and A375 FRT14 to create stable expression cell lines. (A) wild-type IRF-1 is overexpressed from A375 FRT7 conIRF-1 (lane 5) compared to A375 FRT7 (lane 1). Both A375 FRT14 W11R (lane 4) and A375 FRT7 W11R (lane 3) also overexpress mutant IRF-1 compared to empty vector control A375 FRT7 and FRT14 (lane 1 and 2). (B) A downstream target of IRF-1 (ISG-20) is overexpressed as a result of overexpressing wild-type IRF-1 in A375 FRT conIRF-1 (lane 4); all remaining cell lines (lane 1, 2, and 3) show basal levels of ISG-20. (C) Luciferase assay showing TLR3 relative expression to IRF-1 in three different cell lines A375 FRT7, A375 FRT7 W11R, and A375 FRT7 conIRF-1.

4.3.3 Microarray analysis to investigate the panel of IRF-1 regulated targets

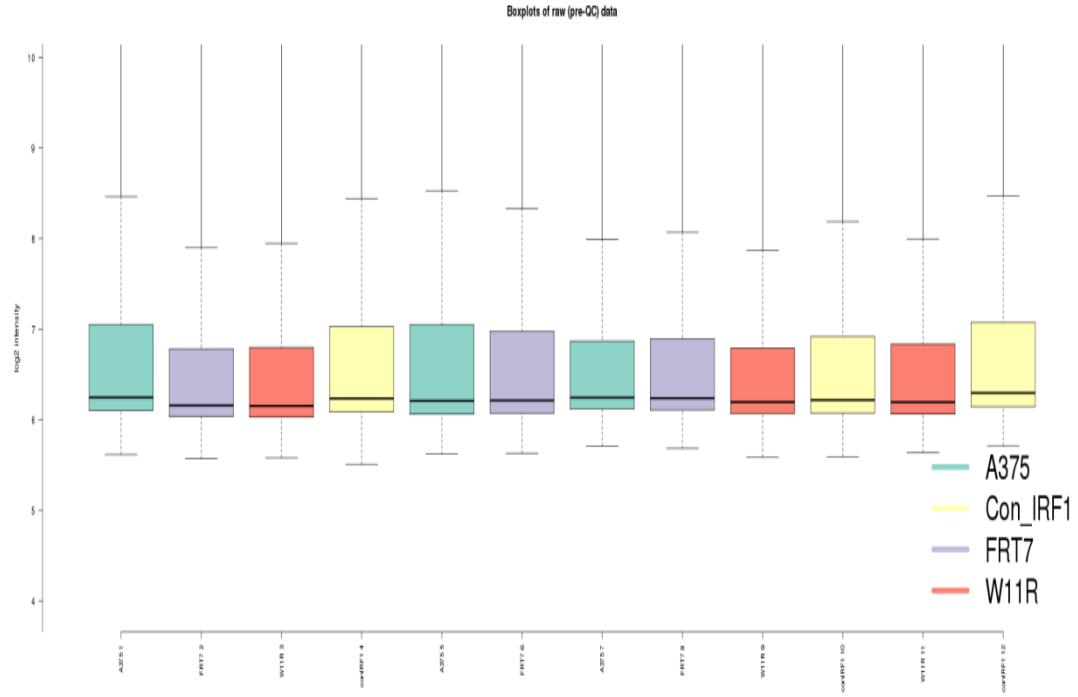
RNA was extracted from A375 wild-type, A375 FRT7, A375 FRT7 W11R and A375 FRT7 conIRF-1 using the RNeasy Mini Kit (Qiagen) and was biotinylated and amplified (cRNA) using the Illumina® TotalPrep RNA Amplification Kit (Applied Biosystems) for hybridization (RNA from each cell line was done in triplicate) making a total of 12 samples. 10µl of cRNA (150ng/µl) from each sample was supplied to the Clinical Research Facility (Western General Hospital, Edinburgh) to quality check cRNA by Agilent 2100 Bioanalyser.

12 samples were hybridized onto Human HT12_V3_0_R1_11283641_A (Illumina), each comprising 48802 features and run on the Illumina Beadstation to give signal intensity for each probe for each sample. Raw data were exported and passed to Fios Genomics (Edinburgh) for microarray data analysis.

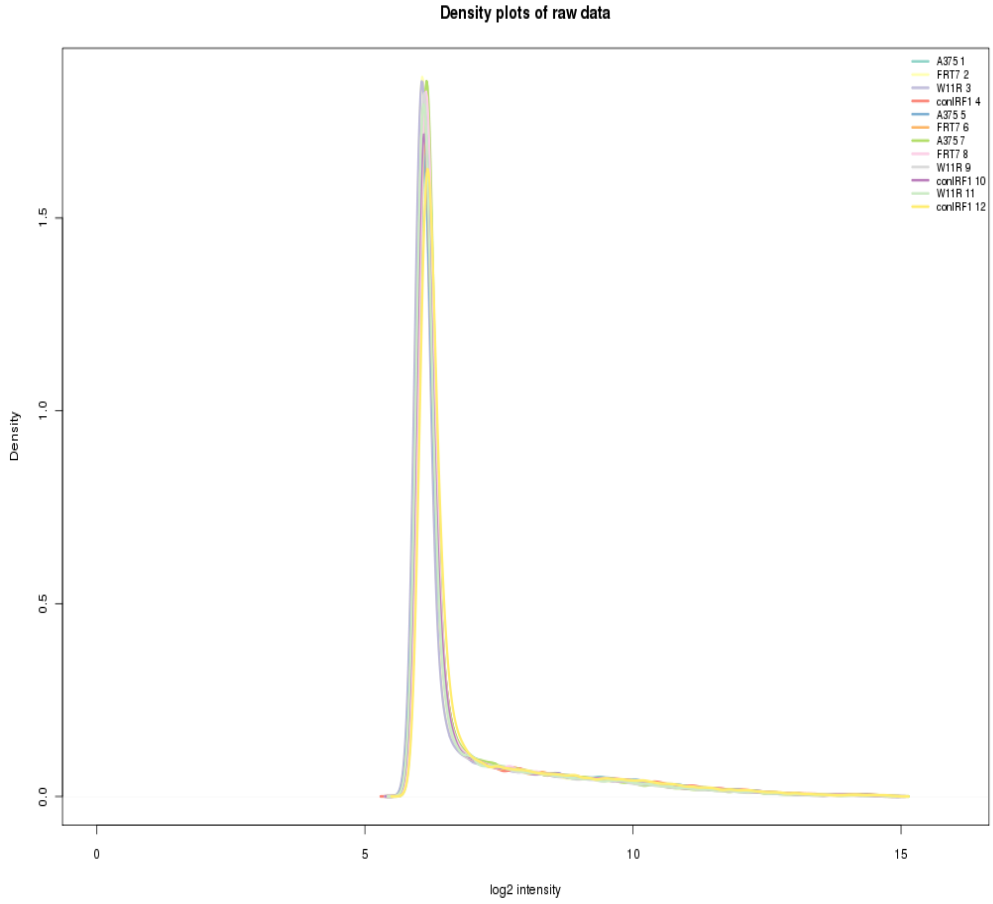
A total of 12 arrays were QC analysed using the Array Quality Metrics package in Bioconductor (Kauffmann *et al*., 2009) Arrays were scored to identify outliers on the basis of 3 metrics, namely MA plot (Figure 4.7.A), Box plot (Figure 4.7.B) and density plot (Figure 4.7.C). However, no outlier(s) has been identified in any 2 out 3 metrics in all replicate for each sample of A375 wild-type, A375 FRT7, A375 FRT7 W11R and A375 FRT7 conIRF-1.


A375 1
FRT7 2
W11R 3
conIRF1 4
A375 5
FRT7 6
A375 7
FRT7 8
W11R 9
conIRF1 10
W11R 11
conIRF1 12

(A)


Boxplots of raw (pre-QC) data
log2 intensity
A375
Con_IRF1
FRT7
W11R

(B)



(C)

Figure 4.7: The Array Quality Metrics Bioconductor QC report. (A) MA Plot for the 12 arrays where M = $\log_2(I_1) - \log_2(I_2)$ and A = 1/2 ($\log_2(I_1) + \log_2(I_2)$). I_1 is the intensity of the array studied and I_2 is the intensity of a "pseudo"-array, which has the median value of all the arrays. Typically, each array is expected to have the mass of the distribution in an MA plot concentrated along the M = 0 axis. (B) Box plot for the 12 arrays, each box corresponds to one array. It gives a simple summary of the distribution of probe intensities across all arrays. Typically, all boxes should have a similar size (IQR) and Y position (median). If the distribution of an individual array is very different from the others, this may indicate an experimental problem. (C) Density plot, Kernel density estimates (smoothed histogram) of data, where each array is represented by a single line. Typically, the distributions should have similar shapes and ranges.

Once the quality check report confirmed that none of the replicates was an outlier, raw data were transferred using a Variance Stabilizing Transformation (VST) method prior to normalisation across all arrays using the Robust Spline Normalisation (RSN) method. Six comparisons: conIRF1 relative to A375, A375 FRT7 relative to A375, W11R relative to A375, conIRF1 relative to A375 FRT7, W11R relative to A375 FRT7, and finally W11R relative to onIRF1 were manually chosen to explore the data. Subsequently, empirical Bayesian analysis was applied, including vertical (within a given comparison) p value adjustment for multiple testing, which controls for false discovery rate. The Bioconductor package limma was used (Smith 2005).

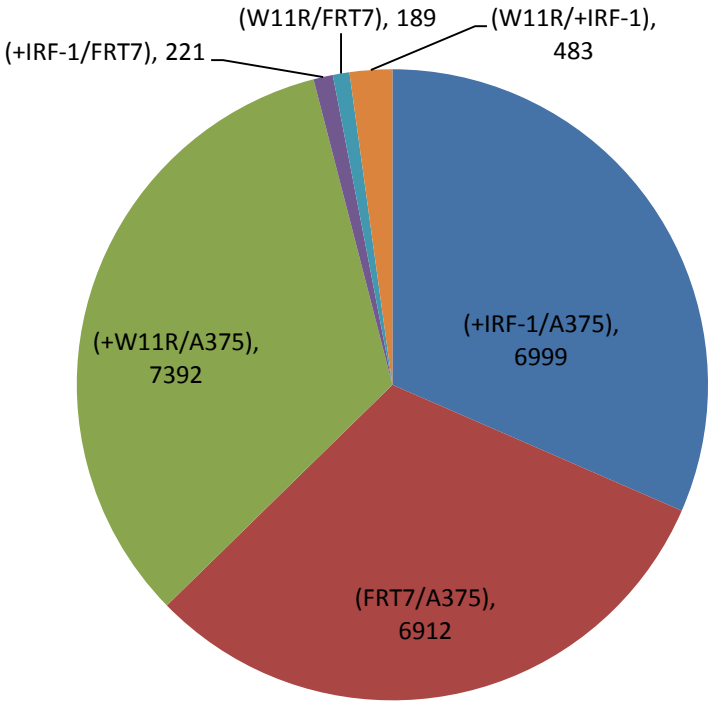


Figure 4.8: Pie chart of all six comparisons undertaken, with their significant array hits. In each case, the (/) sign between the two cell lines translates to "relative to" in common parlance. The number of significant array features at adjusted p≤ 0.05 is shown. Note that for statistical robustness, only those with an adjusted p value ≤ 0.05 should be considered.

The primary output from the empirical Bayesian analysis is fully annotated lists of genes differentially expressed in the comparison of interest. These lists have associated significance statistics, sorted by decreasing order of significance (up to a maximum of 55,000 array features). However, A375 FRT7 conIRF-1 relative to A375 FRT7 gene list with a total of 221 significant hits was illustrated in the Venn diagram (Figure 4.9).

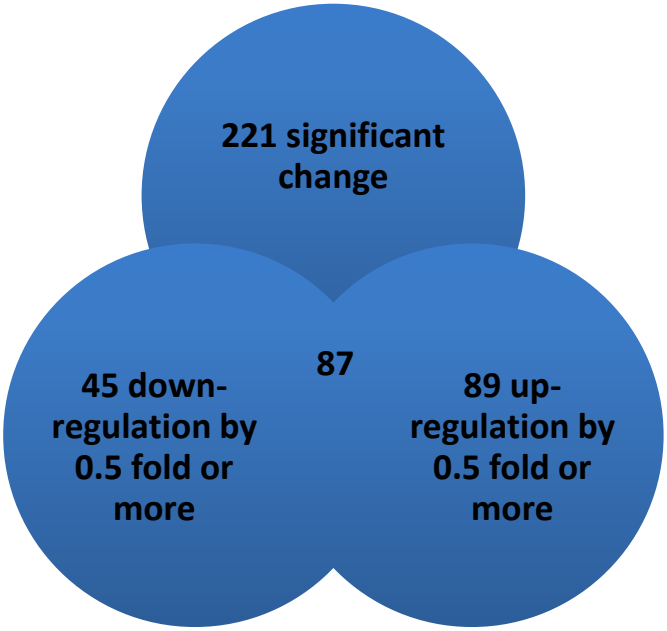


Figure 4.9: Venn diagram illustrating significant array distribution. A total of 221 significant change (p value ≤ 0.05) in A375 FRT7 conIRF-1 relative to A375 FRT7 where 89 arrays were up-regulated by 0.5 fold or more, compared to 45 arrays that were down regulated by 0.5 fold or more. A total of 87 arrays showed either up- or down-regulation but with less than 0.5 fold change.

The A375 FRT7 conIRF-1 relative to A375 FRT7 gene list was checked for potential IRF-1 targets and 12 significant candidates (P value of ≤ 0.0065) showing up-regulation in their expression when wild-type IRF-1, but not W11R IRF-1 mutant, is over expressed were chosen, on the basis of having a role in cancer, cell adhesion, cellular migration, apoptosis, tumour suppressor target genes, cancer therapy, metastatic, cellular adhesion, invasion, and autophagy, for further testing using qRT-PCR.

However, the most statistically significant probe sets may not have a large fold change. Conversely, it is frequently the case that large fold changes are not statistically significant, primarily due to the variances of those probe sets being comparatively great, although subsequent qRT-PCR or other methods may confirm the large fold change values.

Code	Name	Fold(s) Change	Adjusted P value
SORBS2	Sorbin and SH3 domain containing 2	**1.5**	**2.40E-05**
PMEPA1	Prostate transmembrane protein, androgen induced 1	**0.86**	**7.30E-05**
IL24	Interleukin 24	**1.42**	**0.00012**
EGR1	Early growth response 1	**1**	**0.00018**
SPP1	Secreted phosphoprotein 1	**0.8**	**0.00018**
HIPK2	Homeodomain interacting protein kinase 2	**0.62**	**0.00062**
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	**0.68**	**0.00079**
RUNX1	Runt-related transcription factor 1	**0.62**	**0.001**
NEDD4L	Neural precursor cell expressed, developmentally down-regulated 4-like	**0.56**	**0.0028**
SMG1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase	**1.4**	**0.0058**
SH3PXD2A	SH3 and PX domains 2A	**0.66**	**0.0058**
IRF2BP2	Interferon regulatory factor 2 binding protein 2	**0.52**	**0.0065**

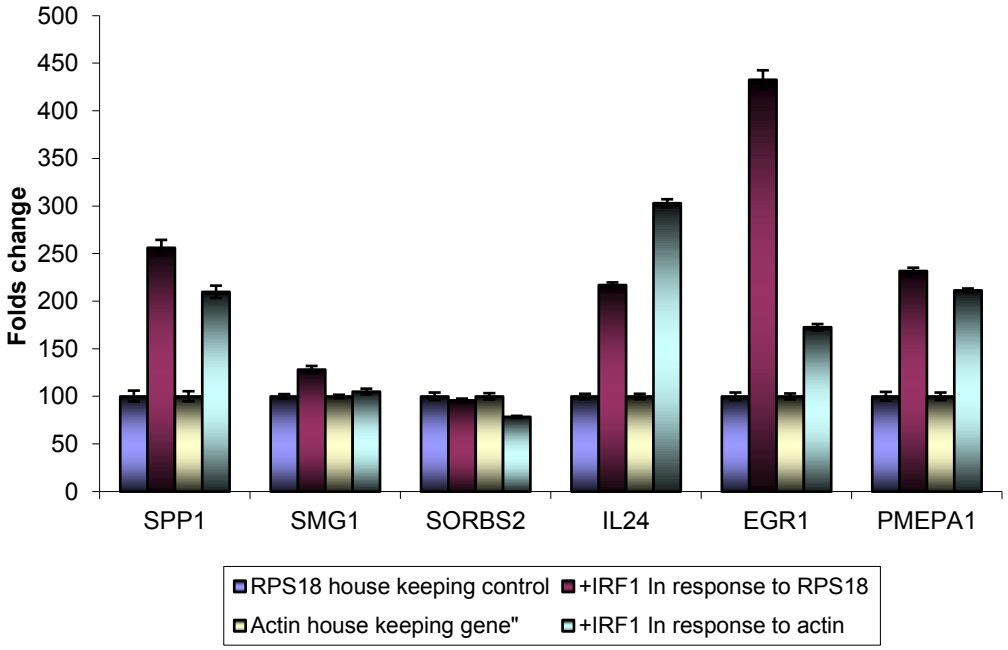
Table 4.1: Twelve potential IRF-1 targets with more than 0.5 fold change in their expression once IRF-1, but not W11R mutant, is over expressed. All candidates are highly significant with a P Value of ≤ 0.0065. Most targets have a role in cancer and cellular adhesion/migration.

4.3.4 Quantitative RT-PCR analysis confirms the role of IRF-1 in the regulation of IRF-1 potential candidates

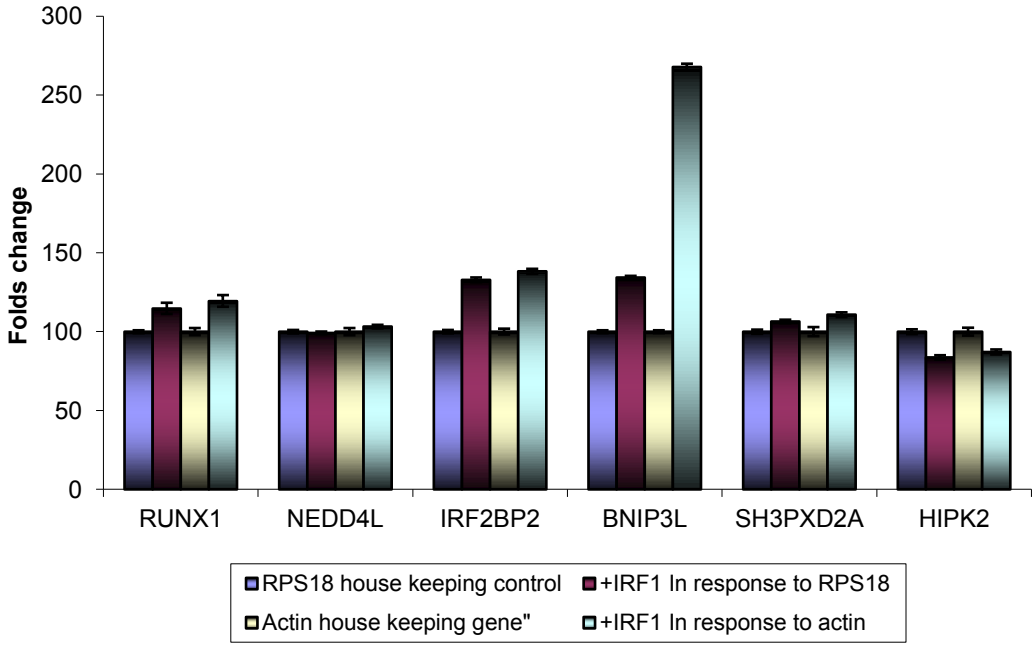
A quantitative real-time PCR analysis of mRNA expression was performed to confirm the differential expression of the selected genes (table 4.1) relative to two house keeping genes *β-actin* and *RPS18*. Primers for qRT-PCR analysis and qRT-PCR master mix were ordered through Dharmacon (Thermo Scientific).

mRNA was extracted from A375 FRT7 and A375 FRT7 conIRF-1 using the RNeasy Mini Kit (Qiagen). 500ng of extracted RNA were reverse transcribed using the Omniscript RT kit (Qiagen) to generate cDNA, which was diluted to make a stock concentration of 25ng/µl. 125ng of cDNA, 6.25µl of PCR water, 1.25µl of Solaris primers, and 12.5µl of Solaris master mix were all mixed together, each sample was treated in quadruplicate for statistical significance. Samples were placed in the qRT-PCR machine using the following thermal cycling program: initial step at 95°C for 15min (enzyme activation) then 40 cycles of 95°C for 15sec (denaturation) and 60°C for 60sec (annealing and extension).

Once qRT-PCR programme was completed, a melting curve for each sample was checked and outliners were eliminated. The Ct value for each replicate sample was noted and mean and standard deviation were calculated for each replicate. ΔCt was calculated by subtracting the Ct (mean) for a house keeping gene from the Ct (mean) of a gene of interest for each condition (A375 FRT7 and A375 FRT7 conIRF-1). ΔΔCt was then calculated by subtracting ΔCt of a house keeping gene from ΔCt of a gene of interest where the value (0) should be in all controls, as subtracting the ΔCt of house keeping gene from itself is always (0). Relative gene expression is then calculated using (1+ primer efficiency) all to the power of −ΔΔCt where primer efficiency is set to be 90%.


500
450
400
350
300
250
200
150
100
50
0
Folds change
SPP1
SMG1
SORBS2
IL24
EGR1
PMEPA1
RPS18 house keeping control
+IRF1 In response to RPS18
Actin house keeping gene"
+IRF1 In response to actin

(A)


300
250
200
150
100
50
0
Folds change
RUNX1
NEDD4L
IRF2BP2
BNIP3L
SH3PXD2A
HIPK2
RPS18 house keeping control
+IRF1 In response to RPS18
Actin house keeping gene"
+IRF1 In response to actin

(B)

Figure 4.10: Quantitative real-time PCR analysis of 12 IRF-1 target genes. (A) First round of screening where 4 target genes SPP1, IL24, EGR1, and PMEPA1 shows clear up-regulation relative to two house keeping genes (actin and RPS18) when IRF-1 is over expressed; SMG1 and SORBS2 show no significant change. (B) Second round of screening where two target genes IRF2BP2 and BNIP3L show clear up-regulation relative to two house keeping genes (actin and RPS18) when IRF-1 is over expressed; RUNX1, NEDd4L, SH3PXD2A, and HIPK2 show no significant change.

4.3.5 *In-silico* analysis of promoter regions

In-silico analysis of the putative promoter region (up to 2 kb upstream to the predicted transcription site) for *IRF2BP2*, *BNIP3L*, *SPP1*, *SMG1*, *IL24*, *EGR1* and *PMEPA1* (all showed high levels of overexpression in qRT-PCR) was carried out using Galaxy https://main.g2.bx.psu.edu/ and UCSC genome browser http://genome.ucsc.edu/index.html?org=Human&db=hg19&hgsid=257732897. DNA sequence was obtained from the UCSC genome browser and uploaded into the Galaxy online tool. Once uploaded, an area of 1000bp or 2000bp upstream of the predicted transcription site was flanked using the "flanking tool". Once flanked, sequence was fetched using the "fetched tool" for a consensus search using Fuzznuc. The analysis revealed all 7 genes have a potential ISRE consensus sequence in their promoter region to which IRF-1 can bind, and a site for putative binding of NFκB binding protein; table (4.2).

Gene	Consensus	Area	Mismatch	Sequence
IRF2BP2	ISRE	Within 1kb	1	[AG]NGAAANNGAAACT
IRF2BP2	NFκB	Within 1kb	1	GGG[AG]NN[CT][CT]CC
BNIP3L	ISRE	Within 2kb	1	[AG]NGAAANNGAAACT
BNIP3L	NFκB	Within 1kb	0	GGG[AG]NN[CT][CT]CC
SPP1	ISRE	Within 2kb	2	[AG]NGAAANNGAAACT
SPP1	NFκB	Within 2kb	1	GGG[AG]NN[CT][CT]CC
SMG1	ISRE	Within 2kb	2	[AG]NGAAANNGAAACT
SMG1	NFκB	Within 1kb	1	GGG[AG]NN[CT][CT]CC
IL24	ISRE	Within 1kb	1	[AG]NGAAANNGAAACT
IL24	NFκB	Within 2kb	1	GGG[AG]NN[CT][CT]CC
EGR1	ISRE	Within 2kb	3	[AG]NGAAANNGAAACT
EGR1	NFκB	Within 2kb	0	GGG[AG]NN[CT][CT]CC
PMEPA1	ISRE	Within 2kb	1	[AG]NGAAANNGAAACT
PMEPA1	NFκB	Within 2kb	0	GGG[AG]NN[CT][CT]CC

Table 4.2: Potential ISRE and NFκB consensus sequences in the promoter region of 7 IRF-1 downstream target genes. Up two 2 kb upstream of the predicted transcription start site was analysed, using the Galaxy online tool, for potential ISRE and NFκB DNA binding motifs with up to 3 mismatches.

4.4 Discussion

IRF-1 is a nuclear transcription factor that regulates a wide range of downstream targets through binding to defined motifs at promoter sites. IRF-1 can induce target genes individually or through association with other cofactors and IRF members, such as IRF-8, creating transcriptional heterocomplexes that bind and regulate various promoters of many genes (Levi *et al*., 2002). To search for unidentified target genes for IRF-1, we overexpressed endogenous IRF-1 rather than stimulating IRF-1, as IRF-1 pathway stimulation might stimulate other signalling pathways leading to up-regulation of non-IRF-1 specific genes. We have developed two isogenic stable cell lines over-expressing wild type IRF-1 and W11R IRF-1, using the Flp-In System™ (Invitrogen).

Host cell lines after FRT integration were tested using Southern blotting to confirm the presence and the number of FRT site(s) (Figure 4.4). Six clones: A375 FRT1, A375 FRT7, A375 FRT8, A375 FRT9, A375 FRT12, and A375 FRT14 have confirmed presence of a single FRT site in their genome by the appearance of single band in their profile digestion (black arrows, Figure 4.4). It is very important in the creation of isogenic stable cell lines to confirm the number of FRT site(s) integrated in the host genome, as the possession of more than one site can lead to non-isogenic creation as one gene of interest could be inserted at one site and a second gene of interest could be inserted at the second site, leading to non-identical expression profiles.

As FRT site integration is random in the cellular genome, IRF-1 pathway integrity was tested after FRT integration to confirm the stability of the IRF-1 pathway. To achieve this, the IRF-1 pathway was stimulated using poly (I:C), a synthetic analogue of double-stranded RNA, to simulate a viral infection through Toll-like receptor 3 (TLR3) and type I interferon (Matsumoto and Seya 2008). All three clones A375 FRT7, A375 FRT12, and A375 FRT14 showed IRF-1 pathway stability, when compared to wild-type A375 (Figure 4.5). Thus, FRT integration does not disturb the IRF-1 pathway in A375 FRT7 A375FRT12, and A375 FRT14.

Once FRT host cell lines had been tested for IRF-1 pathway stability, wild-type IRF-1 and W11R IRF-1 were co-transfected with pOG44 to generate stable cell lines and successful clones overexpressed the desired protein when compared to an empty host cell line (Figure 4.6.A; lane 1, 3, and 5). Protein expression varied from one FRT clone to another due to the fact that FRT integration is random and thus could be affected by chromosomal location and chromatin packing.

To confirm the elimination of DNA binding ability in stable cell lines overexpressing W11R mutant, interferon stimulated gene 20kDa (ISG-20) protein level was used as a marker. ISG-20 has a unique ISRE sequence at its promoter where IRF-1 can bind and initiate transcription once the IRF-1 pathway is induced by interferon type I or II (Congora *et al*., 2000). Only stable cell lines overexpressing wild type IRF-1 managed to induce ISG-20 overexpression (Figure 4.6.B, lane 4), where the basal level of ISG-20 was based on stable cell lines overexpressing W11R mutant, thus mutant IRF-1 (W11R) can not directly bind ISRE. Further testing was also achieved using a luciferase reporter plasmid and the TLR3 promoter (Figure 4.6.C); excessive luciferase activity was only detected in cell lines overexpressing wild type IRF-1, where cell lines overexpressing W11R showed basal levels similar to A375 FRT7 (Figure 4.6.C). Thus, the DNA binding ability is lost in the W11R mutant.

From the six comparisons undertaken by microarray data analysis, the majority of differentially-expressed loci were found in the first three comparisons, namely conIRF-1 relative to A375, A375 FRT7 relative to A375, and W11R relative to A375. This observation suggests that random integrations of FRT loci are disturbing the genomic profile, since the expression profiles of the three constructs (FRT, wild-type IRF-1, and W11R IRF-1) differed quite substantially from the control, and thus attention should primarily be focussed on comparisons four to six (conIRF-1 relative to A375 FRT7, W11R relative to A375 FRT7, and W11R relative to conIRF-1). Within comparisons four to six, 645 array features, representing 566 different genes, were significant in one or more of the comparisons (Figure 4.8).

To identify IRF-1 downstream targets, comparison four (conIRF-1 relative to A375 FRT7) was used. In comparison 4, there are 221 statistically significant arrays containing 89 features that were up-regulated more than 0.5 fold, compared to 45 features that were down-regulated more than 0.5 fold. However, there are 87 features with less than 0.5 fold changes (Figure 4.9). In terms of observed fold change, no features exhibited either more than 2 fold up-regulation or 2 fold down-regulation; fold changes ranged from 1.7 fold up to 1.6 fold down.

Among the genes whose expression was changed by overexpressing wild type IRF-1, we looked for those whose expression was not changed after overexpressing W11R IRF-1. We assumed that the expression observed in the W11R IRF-1 overexpression may result from the absence of wild type IRF-1, implying that these non-induced genes in the case of W11R but induced in the case of wild type IRF-1 are potentially regulated by IRF-1. After analysing the gene lists in comparison 4, we chose to further validate the microarray results for 12 potential IRF-1 targets (table 4.1) using qRT-PCR.

To validate the 12 potential IRF-1 targets chosen from the microarray analysis (table 4.1), qRT-PCR analysis was performed on all 12 genes using the mRNA obtained from cells over expressing IRF-1 (Figure 4.10). Seven genes: *IRF2BP2*, *BNIP3L*, *SPP1*, *SMG1*, *IL-24*, *EGR1*, and *PMEPA1*, showed clear up-regulation at the mRNA level when IRF-1 is over expressed, whereas *RUNX1* and *SH3PXD2A* perhaps a less distinct increase. In addition, *NEDD4L*, *HIPK2*, and *SORBS2* showed either no change or decrease in mRNA level. It is expected that there is variation between microarray and qRT-PCR results, as microarray probes and qRT-PCR primers could have picked two different splice variants, thus *NEDD4L*, *HIPK2*, and *SORBS2* were eliminated from further study.

As mentioned, we have looked at only nine of the many putative genes that are regulated by IRF-1. These genes are involved in key processes taking part in different cellular functions including apoptosis, the immune response, kinase activity, autophagy, cytokine signalling, cell linage differentiation during development, metastasis, tumour growth progression, invasion, and adhesion. The regulatory network for IRF-1 overexpression in melanocytes is illustrated in figure 4.11.

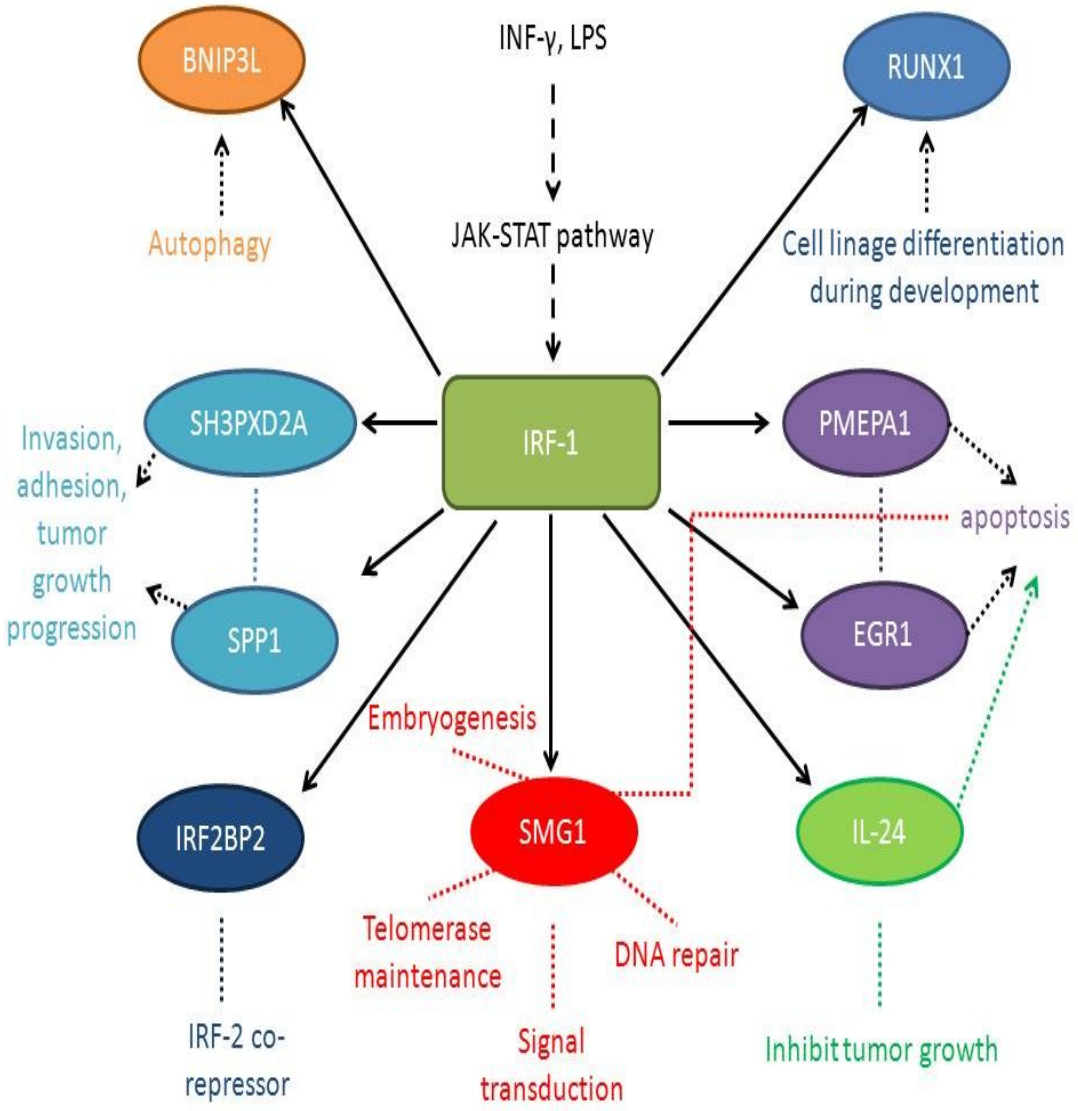


Figure 4.11: Schematic illustration of the IRF-1 regulatory network in melanocytes. Target genes are indicated by oval shapes. Biological function for each target gene is indicated, implying the role of IRF-1 in regulating such function. General IRF-1 stimuli and regulatory pathways are indicated by dashed arrows.

Cell congregation and cell-cell interactions of melanocytes are fundamental events in cancer metastasis and invasion. To become malignant, cancerous cells overexpress metastasis and invasion proteins such as SPP1 and SH3PXD2A. SPP1 expression has been reported to be closely correlated with invasion and metastasis of gastric cancer (Zhang *et al*., 2005). In addition, SPP1 has been reported to be up-regulated and significantly overexpressed in metastatic melanomas and its expression is the first step in melanoma tissue invasion (Zhou *et al*., 2005). Thus, SPP1 is acquired early in melanoma development and progression, and may enhance tumour cell growth in invasive melanomas (Zhou *et al*., 2005).

SH3PXD2A is a scaffolding protein containing SH3 and PX domains. Seals *et al* (2005) reported that SH3PXD2A is required for podosome formation and function and for the protease driven invasion of cancer cells. Podosomes are small, cylindrical adhesive structures containing focal contact proteins, and are found in malignant cells. Cells with reduced SH3PXD2A expression are poorly invasive through Matrigel; SH3PXD2A expression is required in breast cancer and melanoma cells (Courtneidge *et al*., 2005). In addition, Blouw *et al* (2008) confirmed the role of SH3PXD2A in tumour growth *in vivo*.

The role of IRF-1 in regulating the expression of SPP1 and SH3PXD2A is unknown; however, Fu *et al* (2005) reported the hyperexpression of IRF-1 and SPP1 in addition to many genes when a mesangial cell line were stimulated by lipopolysaccharide, although it was not known whether SPP1 overexpression was caused by IRF-1 overexpression or lipopolysaccharide stimulation. Here we have proposed a role for IRF-1 in regulating both SPP1 and SH3PXD2A, suggesting a new role of IRF-1 in cancer cell metastasis and invasion. Further *in-silico* analysis revealed potential binding motifs for IRF-1 and NF-κB in *SPP1* regulatory elements (table 4.2) suggesting that SPP1 might respond to similar signalling cues. It has been reported by Hijiya *et al* (1994) that the *SPP1* promoter region might contain a potential IRF-1 binding site, suggesting a binding motif for IRF-1.

As explained in section 1.4.3.3, IRF-1, as a tumour suppressor gene, plays an important role in apoptosis. IRF-1 has been shown to bind directly to the promoter of many pro-apoptotic genes and thus initiate the process of apoptosis. Among these pro-apoptotic genes, we have identified three distinct apoptotic genes, EGR-1, PMEPA1, and IL-24. EGR-1 protein is an anti-proliferative signal for certain tumour cells and is required for apoptosis induced by stimuli that elevate intracellular Ca^{+2}. To initiate such an apoptotic response, EGR-1 transactivates the promoter region of p53, leading to up-regulation of p53 RNA and protein levels and thus initiating apoptosis (Nair *et al.*, 1997). There is little evidence so far for a link between IRF-1 and p53, however, our data proposes a cooperation between IRF-1 and p53 through EGR-1 in initiating an apoptotic response in melanocytes.

PMEPA1 was originally identified as a highly androgen-induced gene by serial analysis of gene expression in androgen treated prostate cancer cells (Xu *et al*., 2000). Studies on PMEPA1 suggested its role in cell growth regulation, inhibition, and apoptosis (Xu *et al*., 2003; Chen *et al*., 2005). To date, there is no link between IRF-1 and PMEPA1.

IL-24 or melanoma differentiation associated 7 (MDA-7) was originally identified as a tumour suppressor by its activity to supresses cancer growth (Jiang *et al*., 1996) and later it has been shown to have cytokine activity. IL-24 has been identified as a ligand for the JAK1/STAT signalling pathway (Novakova *et al*., 2009) and can induce cancer cell apoptosis, leading to its potential in cancer gene therapy strategies (Zhu *et al*., 2012). Here we identified PMEPA1, EGR-1 and IL-24 as downstream targets of IRF-1 during its apoptotic role. Further *in-silico* analysis (table 4.2) proposed the direct binding of IRF-1 and NF-κB on their regulatory elements through ISRE.

The link between IRF-1 and autophagy has been proposed recently. It has been shown that interferon-γ inhibits cellular growth of hepatocellular carcinoma by non-apoptotic cell death (Li *et al*., 2012). In addition, interferon-γ induces autophagosome formation, where IRF-1 overexpression also induces autophagy in hepatocellular carcinoma (Li *et al*., 2012). A further study proposed a novel role of IRF-1 and nitric oxide in the regulation of macrophage autophagy during lipopolysaccharide stimulation (Zhang *et al*., 2012). In addition, IRF-1 has been shown to regulate both immune cell apoptosis and autophagy in a murine endotoxemia model (Zhang *et al*., 2012). BNIP3L has been reported to be associated with autophagy (Velde *et al*., 2000) and it has been shown that arsenic trioxide induces autophagic cell death, but not apoptosis, and the induction of apoptosis in gliomoa cells is mediated by the mitochondrial cell death protein BNIP3 and its homologue BNIP3L (Kanzawa *et al*., 2005). A further role of BNIP3 proteins in autophagy, cell death, and mitophagy has been described (Zhang *et al*., 2009; Youle *et al*., 2011). The mechanism of IRF-1 regulation of autophagy is not fully characterized; here we proposed the direct binding of IRF-1 to the regulatory element of BNIP3L to facilitate autophagy.

RUNX1 is a transcription factor that regulates critical processes in many aspects of haematopoiesis (Lam *et al*., 2012). Many haematological diseases, such as myelodysplastic syndrome and acute myeloid leukaemia, have been associated with mutations in *RUNX1* (Lam *et al*., 2012). To date, there is no link between IRF-1 and RUNX1 in the literature apart from the observation that both are mutated in some acute myeloid leukaemias. Here we also propose the direct binding of IRF-1 to a RUNX1 regulatory element, as the *in-silico* analysis (table 4.2) revealed the potential of acquiring an ISRE binding motif.

SMG-1 is a gene involved in nonsense mediated mRNA decay as part of the mRNA surveillance complex (Yamashita *et al*., 2001). This involvement is through the phosphorylation of specific serine residues in major components of the surveillance complex (Yamashita *et al*., 2001). Thus, SMG-1 has a kinetic biological role. Gehen *et al* (2008) reported the direct phosphorylation of p53 at serine 15 by SMG-1, suggesting that SMG-1 is a proximal regulator of DNA damage signalling (Gehen *et al*., 2008).

Here we propose a further cooperation between IRF-1 and p53, which might be mediated by SMG-1 during the DNA damage response. SMG-1 has been reported to regulate other biological roles including DNA repair, telomerase maintenance, embryogenesis, and apoptosis (Azzalin *et al*., 2006 and Cheung *et al*., 2011). SMG-1 regulation by IRF-1 has broadened the biological role of IRF-1 to cover many other unreported roles of IRF-1. Finally, it is known that IRF-2 is a well-characterized repressor of IRF-1 (Harada *et al*., 1989), where IRF2BP2 is also co-repressor of IRF-2 (Childs and Goodbourn, 2003), suggesting that activation of IRF2BP2 might be controlled by IRF-1.

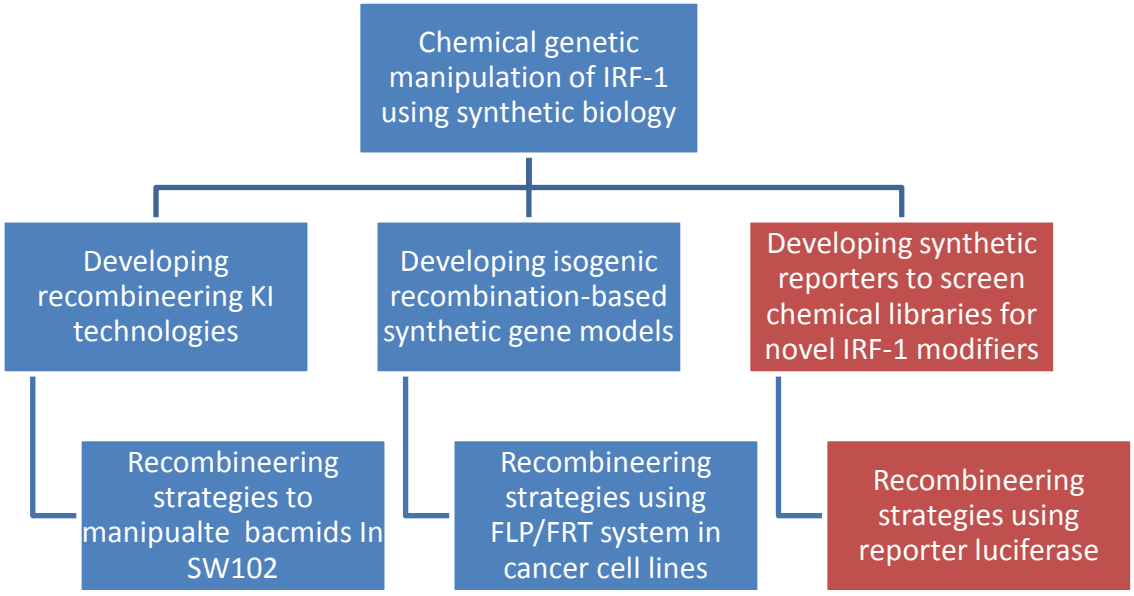
To reveal the role of these potential IRF-1 targets in signalling pathways, KEGG pathway functional enrichment analysis was undertaken for comparison four. Analysis shows 25 KEGG pathways were statistically enriched (appendix). Pathways enriched in up-regulated loci include those involved in Toll-like receptor signalling, prion disease, bladder cancer, small cell lung cancer, and the p53 signalling pathway; however, pathways enriched in down-regulation loci include the vascular endothelial growth factor signalling pathway and the mitogen-activated protein kinase signalling pathway.

This chapter outlined the creation of two isogenic stable cell lines overexpressing either wild-type IRF-1 or W11R IRF-1. Both cell lines were created to screen potential IRF-1 targets using microarray technology. We proposed new biological roles, which can be regulated by IRF-1 in melanocytes, including cellular invasion, adhesion, tumour growth metastasis, embryogenesis, DNA repair, and telomerase maintenance. In addition, we also validated current known biological roles of IRF-1 including apoptosis, autophagy, and tumour growth inhibition.

Chapter 5: Developing synthetic reporters to screen chemical libraries for novel IRF-1 modifiers

5.1 Chapter abstract

Luciferase reporter assays have been used to analyse the activity of many transcriptional regulator elements. However, luciferase assays involve the transient transfection of a luciferase reporter plasmid containing a regulatory element into a chosen host cell line, and this can cause difficulties when large chemical libraries need to be screened. Thus, this chapter describes the development of a synthetic stable reporter cell line that can be used where large scale screening is needed. This developed stable reporter cell line was subsequently used to screen a kinase inhibitor library, which has revealed some new IRF-1 regulation pathways.



5.2 Chapter Introduction

High throughput screening (HTS) of chemical libraries against biological targets is a key requirement in the pharmaceutical industry. High quality screening assay methods are needed to translate specific bio-molecular phenomena into observable parameters detected using radioactivity, photon absorption or photon emission (Fan and Wood 2007). By far, the most dominant methodological assay in designing HTS tool is photon emission due to its speed, accuracy, sensitivity, and broad adaptability to biological targets (Minor, 2006). This chapter will outline the steps used in creating a reporter stable cell line that can be used for HTS. In addition, the reporter cell line was used to screen a kinase library to identify potential new IRF-1 modifiers.

5.2.1 Interferon signal transduction

Interferon signalling involves membrane receptors designed either for class 1 interferon (INF-α or β) or class II interferon (INF-γ). Membrane receptors for class I interferon are composed of IFNAR1 and IFNAR2 and membrane receptors for class II interferon are composed of two pairs of IFNGR1 and IFNAR2 (Figure 5.1). Both types of receptors consist of transmembrane protein subunits with an extracellular and an intracellular domain.

When class I receptors are stimulated by interferon-α or -β, the Janus family of protein tyrosine kinases (Jak1) and (Tyk2) (located near the intracellular domain of IFNAR1 and IFNAR2) become activated, followed by site-specific tyrosine phosphorylation of STAT1 and STAT2 (Darnell *et al*., 1994) (Figure 5.1). The resultant phosphorylated STAT1 and STAT2, in combination with IRF-9, form a heterotrimeric transcription factor complex named as ISGF3. This binds to ISRE consensuses sequences at the promoter of an interferon-induced gene to initiate its transcription (Darnell *et al*., 1994).

In addition, the phosphorylated STAT1 also undergoes homo-dimerization to form an active transcription factor named interferon-γ activated factor/interferon-α activated factor (GAF/AAF) that can bind to GAS consensus sequences (TTCNNNGAA) and activate the transcription of IRF-1 and/or other interferon-induced genes (Darnell *et al*., 1994) (Figure 5.1).

In the case of the class II receptor, interferon-γ stimulates the dimerization and phosphorylation of IFNGR1 by Jak1 and IFNAR2 by Jak2 (Ihle, 1995) resulting in efficient STAT1 activation (Darnell *et al*, 1994); however, STAT2 can also become tyrosine phosphorylated after interferon-γ stimulation to form ISGF3 (not shown in Figure 5.1), but at a lower level compared to interferon-α/β stimulation (Takaoka *et al*., 2000).

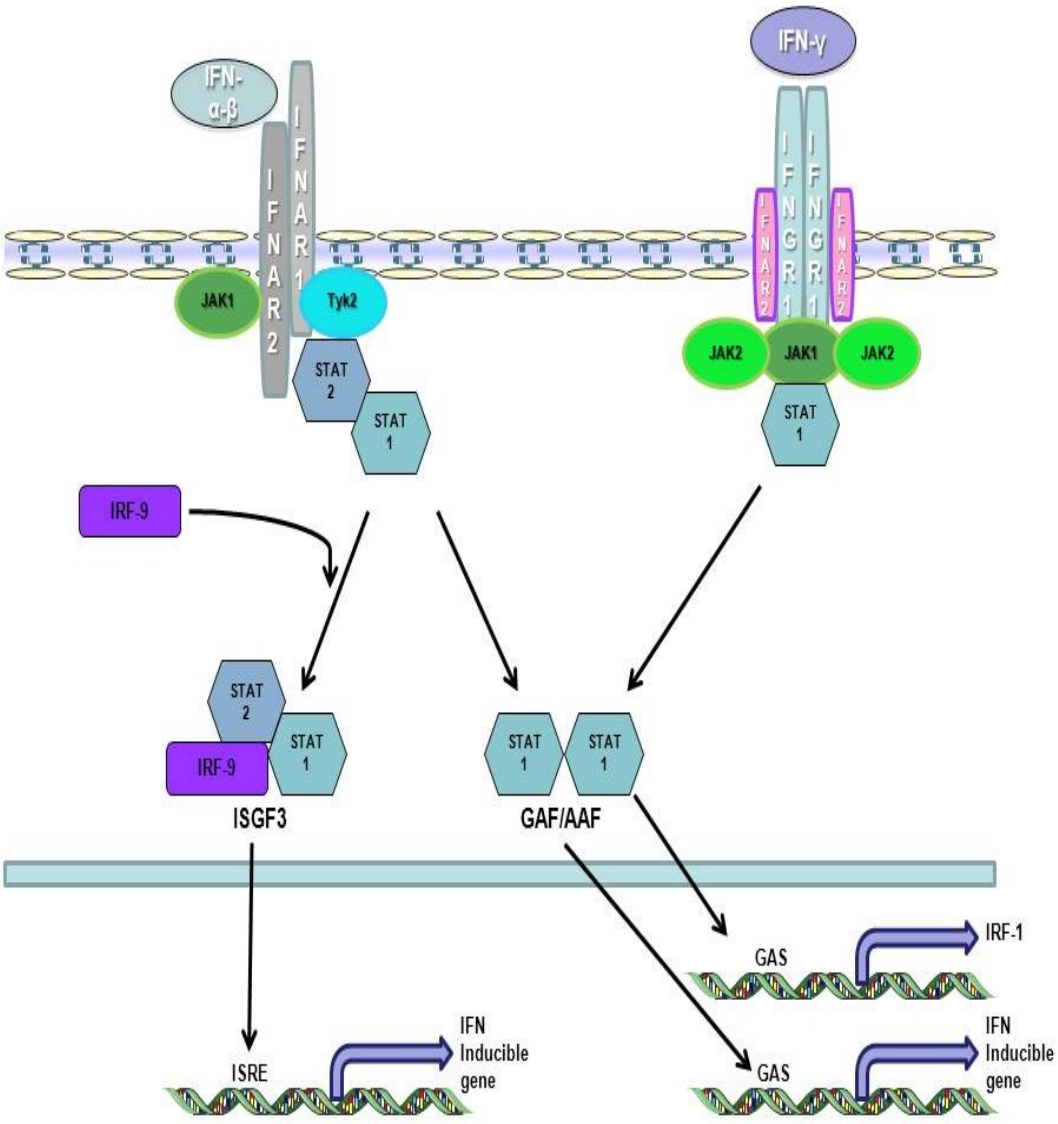


Figure 5.1 Signal transduction pathways and cross talk in the interferon system. Interferon signal transduction involves a set of protein called tyrosine kinases belonging to the JAK protein family, which are important in phosphorylation process. Also, STAT proteins play roles in as signal transducers and as transcription factors by initiating the transcription of interferon-inducible genes.

5.2.2 Bioluminescence

Bioluminescence is a form of chemiluminescence, which can be found in many living organisms such as jellyfish, fireflies, and sea pansies. It is an enzyme-catalysed process, where the enzymes are luciferases and the substrates are luciferins. In the firefly (*Photinus pyralis*), luciferase is a 61kDa monomeric enzyme, which does not require any post-translational modification for activity (De Wet *et al*., 1987). The mechanism involves combining luciferins with ATP to form luciferyl–AMP (Figure 5.2), which then reacts with oxygen to create another enzyme-bound intermediate in a high-energy state known as oxyluciferin. This intermediate, oxyluciferin, then emits energy in the form of yellow-green light with a spectral maximum of 560nm (Fan and Wood 2007).

A different luciferase system can be found in sea pansy (*Renilla reniformis*) which is again a monomeric enzyme of 36kDa protein size that catalyses the oxidation of coelenterazine to yield coelenteramide and blue light with a spectral maximum of 480nm (Lorenz *et al*., 1991). Since both systems have different spectra at which light is emitted, the *Renilla* system is often used to co-report in conjunction with the firefly system to check for experimental and reading errors.

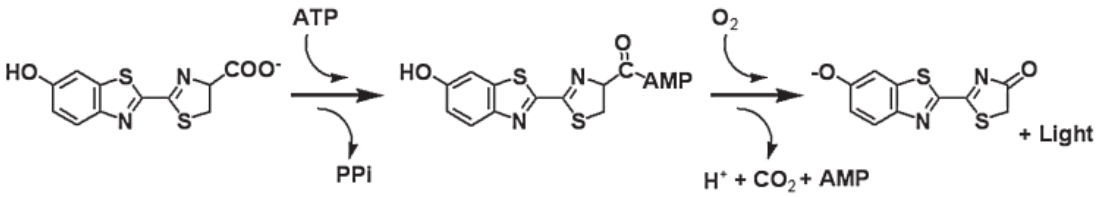


Figure 5.2: The firefly luciferase system. Briefly, luciferase catalyses the interation of luciferins with ATP to form luciferyl-AMP which then reacts with oxygen to create oxyluciferin, which then loses the excess energy in the form of light.

Light intensity from this type of reaction is used to represent and correlate biological parameters. This is achieved by holding two of the three reaction components (ATP, enzyme, and substrate) constant where the variable component can be correlated with the process of interest. Depending on the bioluminescence assay, the variable competent may be ATP, enzyme, or substrate. In this chapter, we develop a genetic reporter stable cell line that has luciferase enzyme concentration as a variable where, all other components are kept constant.

Many applications used for HTS are based on genetic reporters, as the drug discovery process is associated with cellular events and genetic regulation. By cloning a regulatory element of choice (e.g. known promoter, portions of promoter, or known response and enhancer elements) upstream of a gene encoding luciferase, the cellular events resulting from a cloned element can be easily detected by a luminescent signal.

As genetic reporters involve the transfection of a plasmid carrying the luciferase gene regulated by a chosen element, it is necessary to incorporate an internal control to minimise false hits resulting from variations in cell number or transfection technique, to achieve good data quality. An internal control could be a second luciferase system such as the *Renilla* luciferase co-transfected with the firefly luciferase system. However, introducing both systems requires a standard two plasmid approach where the first plasmid contains the chosen regulatory element and firefly luciferase gene and the second plasmid contains a constitutive promoter (SV40) controlling the *Renilla* luciferase gene.

A dual luciferase reagent can then be used to measure signals of firefly and *Renilla* luciferases in the same sample. Readings from firefly luciferase could be normalised to the *Renilla* signal, to eliminate cell number and transfection errors. In most cases of HTS, there is no need for a transfection control as screening of chemical libraries is done using stable reporter cell lines where plasmid transfection is eliminated, thus one luciferase system, such as firefly, should be sufficient.

Previous studies in our lab have revealed that one of the best choices to analyse IRF-1 transcriptional activity using a luciferase assay is to use the Toll-like receptor 3 regulatory element. Thus, the IRF-1 genetic reporter stable cell line contains the toll-like receptor 3 regulatory element. This cell line was created, using the Flp-In System™ (chapter 4) and recombineering technology (chapter 3), by K. Belz under my direct supervision. A kinase inhibitor toolbox (Tocriscreen^{TM}) containing 80 different kinase inhibitors was screened using the IRF-1 transcription reporter cell line to identify potential new IRF-1 modifiers.

5.3 Methods and results

5.3.1 Creating IRF-1 reporter cell lines using the Flp-In™ System and recombineering technology

A pGL3 luciferase reporter vector (Promega) containing 588bp of the human TLR3 regulatory element (pGL3-hTLR3) was kindly given by Dr. Michael Rehli (University of Regensburg, Germany). The pGL3-hTLR3 was used as a PCR template to amplify a 2.3kb fragment containing the hTLR3 regulatory element and the firefly luciferase gene flanked by attB1 and attB2 Gateway® Recombination sites. Primers used in the PCR reaction were:

Forward	5′-GGGGACAAGTTTGTACAAAAAAGCAG GCTTCGAAGGAGATAGAACCATGGGTA CCGAGCTCTTACGC-3′
Reverse	5′-GGGGACCACTTTGTACAAGAAAGCTG GGTCCTAGTCGACGGATCCTTATCG-3′

Yellow: Gateway® recombineering sequence
Green: Shine-Dalgarno sequence
Blue: Kozak sequence
Pink: Start
Red: Stop
Grey: PCR primers

PCR product was then cloned into pDONR™221 (Invitrogen) using a BP reaction and successful clones were profile digested and sequenced to validate the successful cloning of PCR product. Once confirmed, an LR reaction between pDONR™221 and pEF5/FRT/V5-DEST (chapter 3) was performed to clone the TLR3-luciferase cassette into the FRT vector and clones were profile digested and sequenced to confirm fragment integration. The IRF-1 reporter cell lines were created by co-transfecting pOG44 and the FRT expression vector into A375-FRT7 (chapter 3) using Attractene, after which, cells were challenged with hygromycin for two weeks. Successful clones were tested for their luciferase activity; however, luciferase activity was undetectable (data not shown).

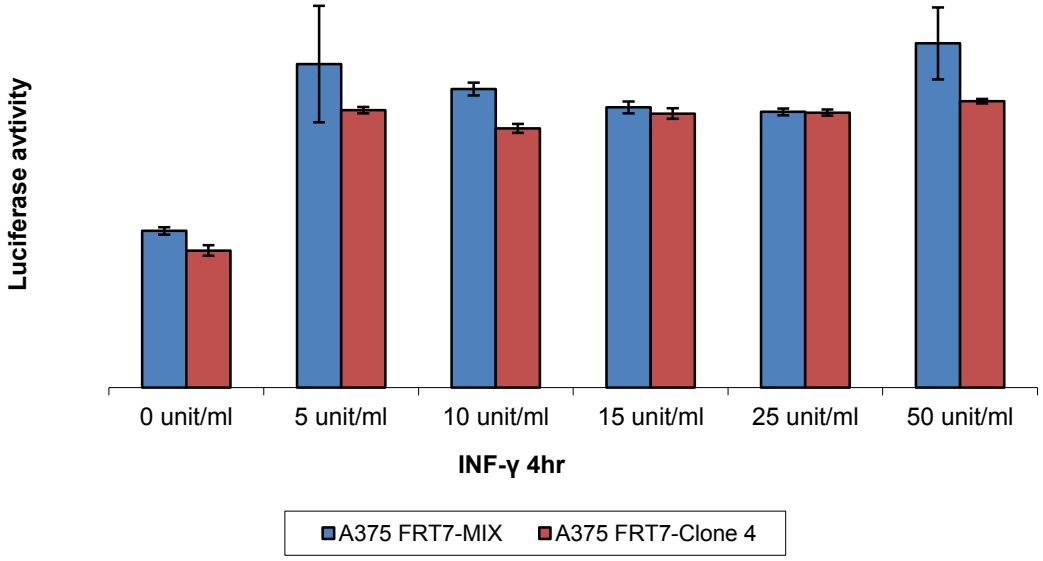
To create reporter cell lines using recombineering, the pGL3-hTLR3 reporter vector needed to have a selectable marker useable in eukaryotes. Thus, the kanamycin/neomycin selectable marker needed to be cloned into pGL3-hTLR3. PCR primers (dark grey) with homologous arms (light grey) were designed to amplify kanamycin/neomycin with PGK and EM7 regulatory elements from pLox-STOP-Lox-β-catenin (Figure 3.11, chapter 3) and recombine the product into pGL3-hTLR3 reporter vector at an exact location just after the luciferase polyA signal. Successful recombination caused the loss of a unique *BamHI* site at pGL3-hTLR3.

Forward	5′-GAGGTTTTTTAAAGCAAGTAAAACCT CTACAAATGTGGTAAAATCGATAAAATT CTACCGGGTAGGGGAG-3′
Reverse	5′-CCGCGCCCACCGGAAGGAGCTGACTG GGTTGAAGGCTCTCAAGGGCATCGTCAG AAGAACTCGTCAAGAA-3′

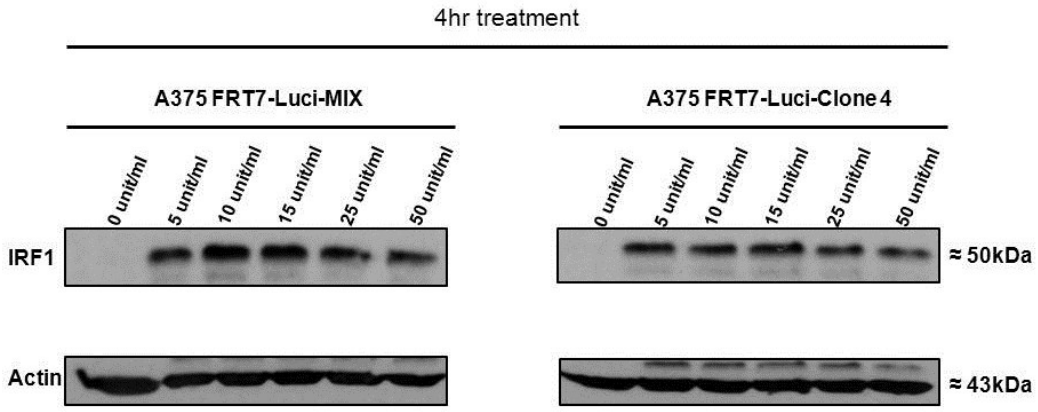
pLox-STOP-Lox-β-catenin was linearized by *ZraI* followed by *DpnI* digestion to minimise false colony formation post PCR reaction. Digested pLox-STOP-Lox-β-catenin (PCR template) was diluted and used in the PCR reaction. Each PCR cycle was performed at 95°C for 1min (denaturation step) followed by 64°C for 30sec (annealing step) followed by 72°C for 3min (elongation step) for 30 cycles. PCR product was purified using PCR clean-up kit (Qiagen) and run on 1% agarose gel to check the size. Once the PCR product was confirmed, a combination of 20ng of pGL3-hTLR3 and the cleaned PCR product (100ng, 150ng, and 200ng) were electroporated in heat-induced and -un-induced (negative control) SW102 for recombination.

Following recombination, cells were allowed to recover for 1hr at 32°C without antibiotic, then fresh LB containing ampicillin and kanamycin was added and cells were grown overnight at 32°C. Tubes with positive growth were taken for miniprep of DNA (Qiagen), which was digested with *BamHI* and 0.1ng of plasmid DNA was re-electroporated into fresh SW102, to minimise the introduction of multiple copy numbers. The resulting vector pGL3-hTLR3-Luci was sequenced verified to confirm successful recombination.

A375-FRT7 cells were transfected with pGL3-hTLR3-luci using Attractene and 24hr post transfection cells were challenged with geneticin (G418) for two weeks, or until complete death of a no DNA control. Single clones where picked and named as A375-FRT7-Luci-clone 4; mixed clones were named as A375-FRT7-Luci-MIX. To test picked clones for luciferase activity, A375-FRT7-Luci-clone 4 and A375-FRT7-Luci-MIX were seeded on 6-well tissue culture plates. The following day, cells were treated with a titration of INF-γ for 4hr and 24hr. IRF-1 transcriptional activity and protein level were measured by luciferase assay and immunoblotting (Figure 5.3) and (Figure 5.4). Results show both clones A375 FRT7-Luci-MIX and A375 FRT7-Luci-clone 4 have acquired luciferase activity; however, A375-FRT7-Luci-MIX seems to have a better luciferase signal than A375-FRT7-Luci-clone 4. In addition, IRF-1 protein levels correlate with these findings as a result of INF-γ treatment. Thus, A375 FRT7-Luci-MIX was chosen clone for HTS.

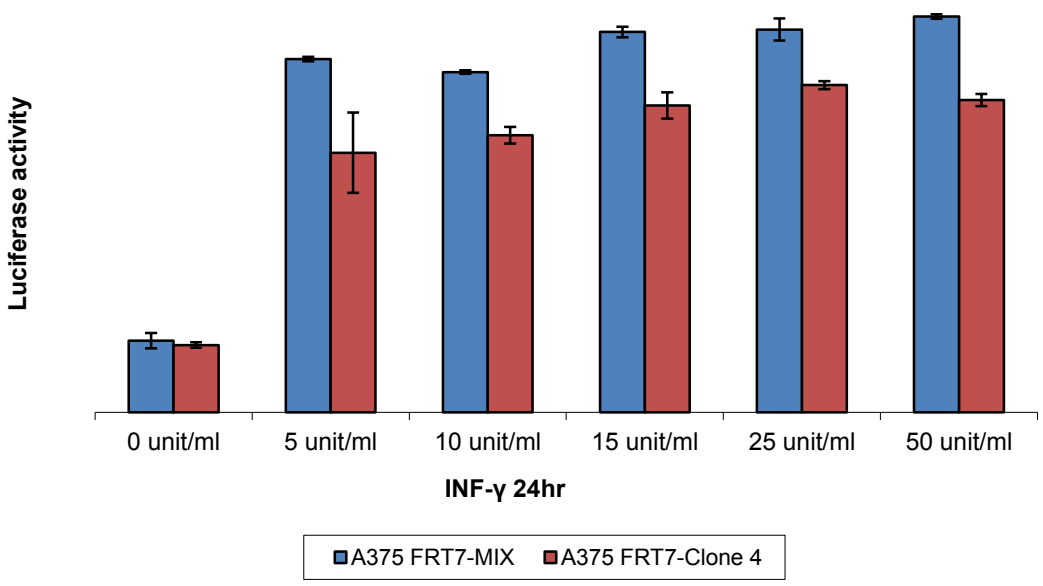


(A)

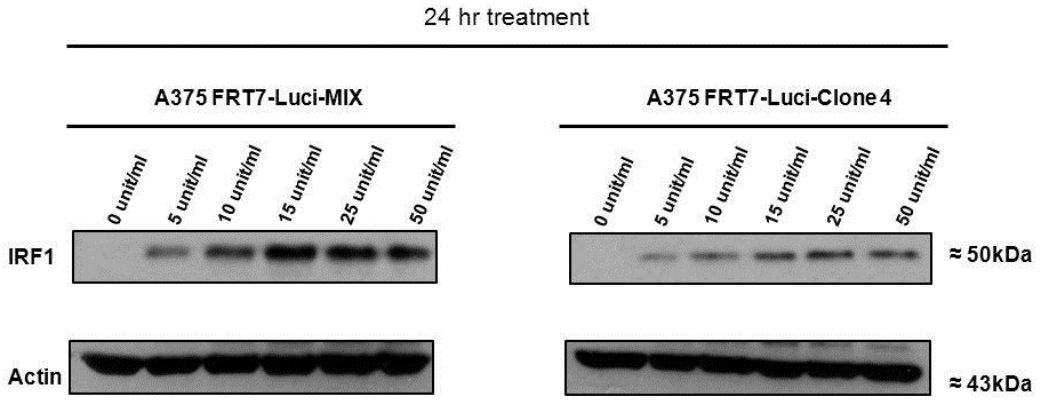


(B)

Figure 5.3: IRF-1 transcriptional activity in A375 FRT7-Luci-MIX and A375 FRT7-Luci-clone 4. (A) Reporter cell lines were treated with a titration of INF-γ for 4hr followed by measurement of IRF-1 transcriptional activity using luciferase. (B) IRF-1 protein levels, post INF-γ treatment, were detected using immunoblotting.



(A)



(B)

Figure 5.4: IRF-1 transcriptional activity in A375 FRT7-Luci-MIX and A375 FRT7-Luci-clone 4. (A) Reporter cell lines were treated with a titration of INF-γ for 24hr followed by measurement of IRF-1 transcriptional activity using luciferase. (B) IRF-1 protein levels, post INF-γ treatment, were detected using immunoblotting.

5.3.2 High throughput screening of Tocriscreen™ kinase inhibitor library using A375 FRT7-Luci-MIX

A375 FRT-Luci-MIX cells were seeded onto 96-well plates 18hr prior to treatment. The following day, cells were treated with eight different concentrations (50, 25, 12.5, 6.25, 3.1, 0.8, and 0.4μM) of each kinase inhibitor for 24hr, after which cells were washed three times with PBS and luciferase activity was measured.

Results revealed 40 different kinase inhibitors caused a decrease in IRF-1 transcriptional activity whereas eight kinase inhibitors caused an increase in its activity; the remaining 32 inhibitors showed no significant change in IRF-1 transcriptional activity (data not shown). The library was decoded and six kinase inhibitors were picked and short-listed for further analysis (table 5.1).

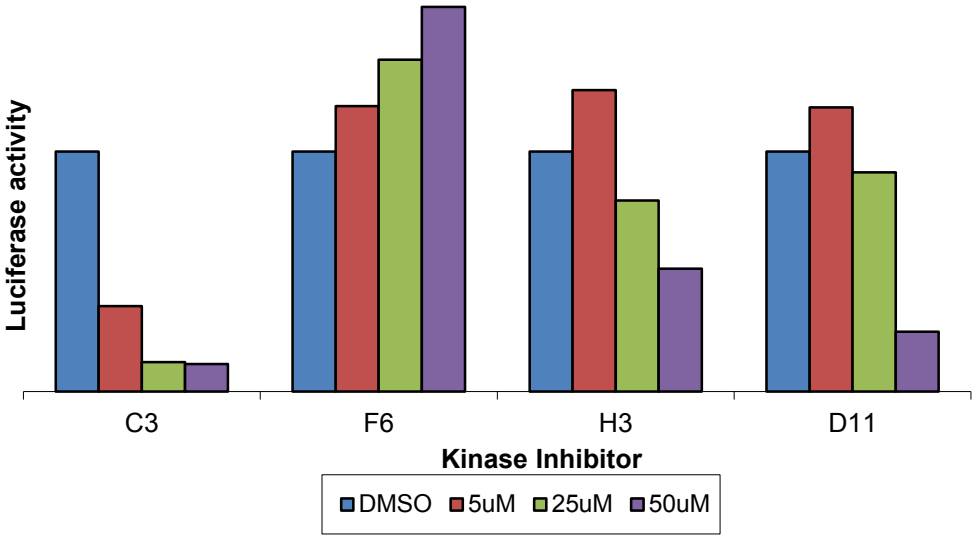
Code	Target	Description	Effect
H3	*EGFR*	Orally active, selective EGFR inhibitor	Repress
C3	*VEGFR*	Potent inhibitor of VEGFR tyrosine kinase	Repress
E4	*EGFR*	Highly selective EGFR kinase inhibitor	Repress
E9	*VEGFR*	Potent, selective inhibitor of VEGFR2	Repress
F6	*FLT3*	Potent FLT3 inhibitor	Stimulate
D11	*CK2*	Selective cell-promoter Casein Kinase 2 inhibitor	Stimulate

Table 5.1: Six kinase inhibitors were short-listed for further analysis. A375 FRT-Luci-MIX cells were seeded onto 96-well plates. Cells were treated with eight different concentrations for each kinase inhibitor for 24hr. The following day cells were washed three times with PBS and luciferase activity was measured. Four (H3, C3, E4, and E9) showed a decrease in IRF-1 transcriptional activity by luciferase reporter, and two (F6 and D11) showed an increase.

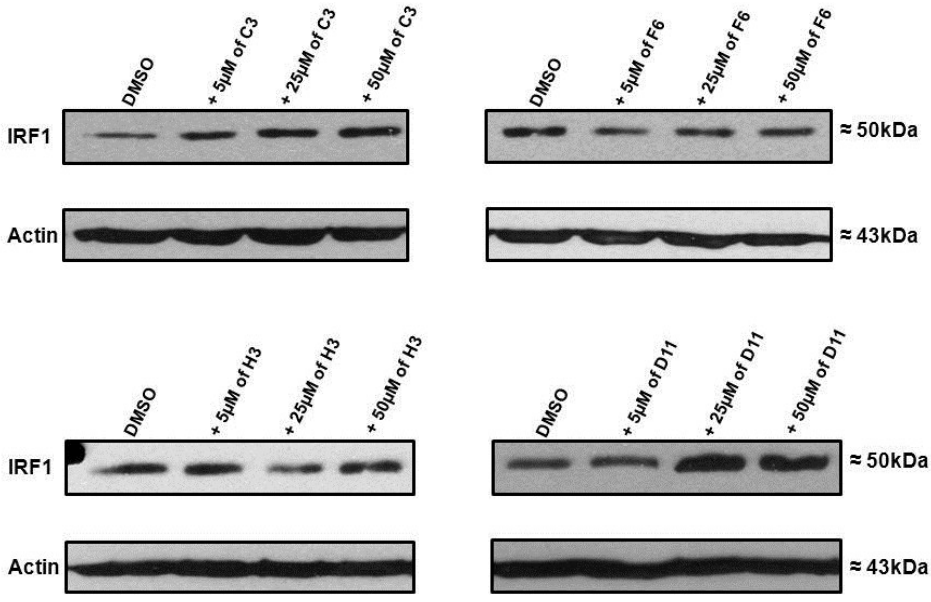
The six short-listed kinase inhibitors were subjected to a second round of screening to confirm the reproducibility of the results. A375 FRT7-Luci-MIX cells were seeded onto 96-well plates 18hr prior to treatment. The following day cells were treated with seven different concentrations (50, 25, 12.5, 6.25, 3.1, 0.8, and 0.4μM) for each kinase inhibitor for 24hr. Following treatment, cells were washed three times with PBS and luciferase activity was measured. Results revealed that C3 and F6 showed reproducible results similar to the first rounds, whereas E9 and D11 results were not reproducible and thus were eliminated from further study. However, other kinase inhibitors, such as E4, and H3, show reproducible effects but only at high concentrations i,e 25 and 50μM (data not shown).

To validate outcomes from round II and to identify minimum inhibitory concentrations and minimum inhibitory times required for each of the six short-listed kinase inhibitors, a third screening round was done in which A375 FRT7-Luci-MIX cells were treated with 4 different concentrations (50, 25, 12.5, and 6.25μM) for each kinase inhibitor for 2, 4, 8, 24, and 48hr. Following treatment cells were washed with PBS 3 times and luciferase activity was measured. The third round of screening (data not shown) revealed that results for some kinase inhibitors (D11 and E9) were not reproducible (compared with round II) and thus they were eliminated from further studies. However, some kinase inhibitors (H3 and E4) showed reproducible results but their effect was only noticeable at high concentrations (25 and 50μM) and after long treatment times (24hr or 48hr). F6 and C3 showed a reproducible pattern even at low concentrations (data not shown).

To test whether these kinase inhibitors have an effect on IRF-1 protein levels, A375 FRT7-Luci-MIX cells were seeded onto 6-well tissue culture plates 18hr pre treatment; the following day, cells were treated with three different concentrations (5, 25, and 50μM) for each kinase inhibitor (C3, F6, H3, and D11) for 24hr. Following treatment, protein lysate was subjected to immunoblotting and luciferase activity measurement (Figure 5.5). Results show that the effects of C3 are reproducible even at low dosage (5μM) where F6 still stimulates IRF-1 transcriptional activity and H3 has effects at high dosage only (50μM). There was no change in IRF-1 protein level as a result of kinase inhibition.



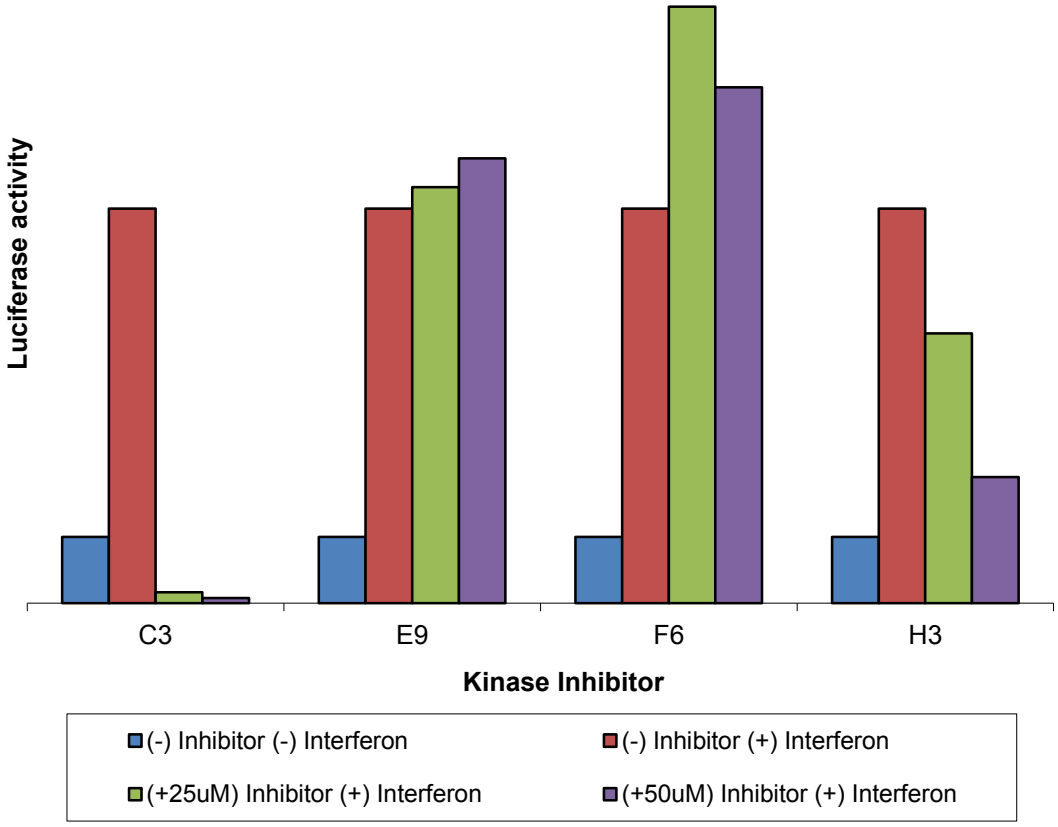
(A)



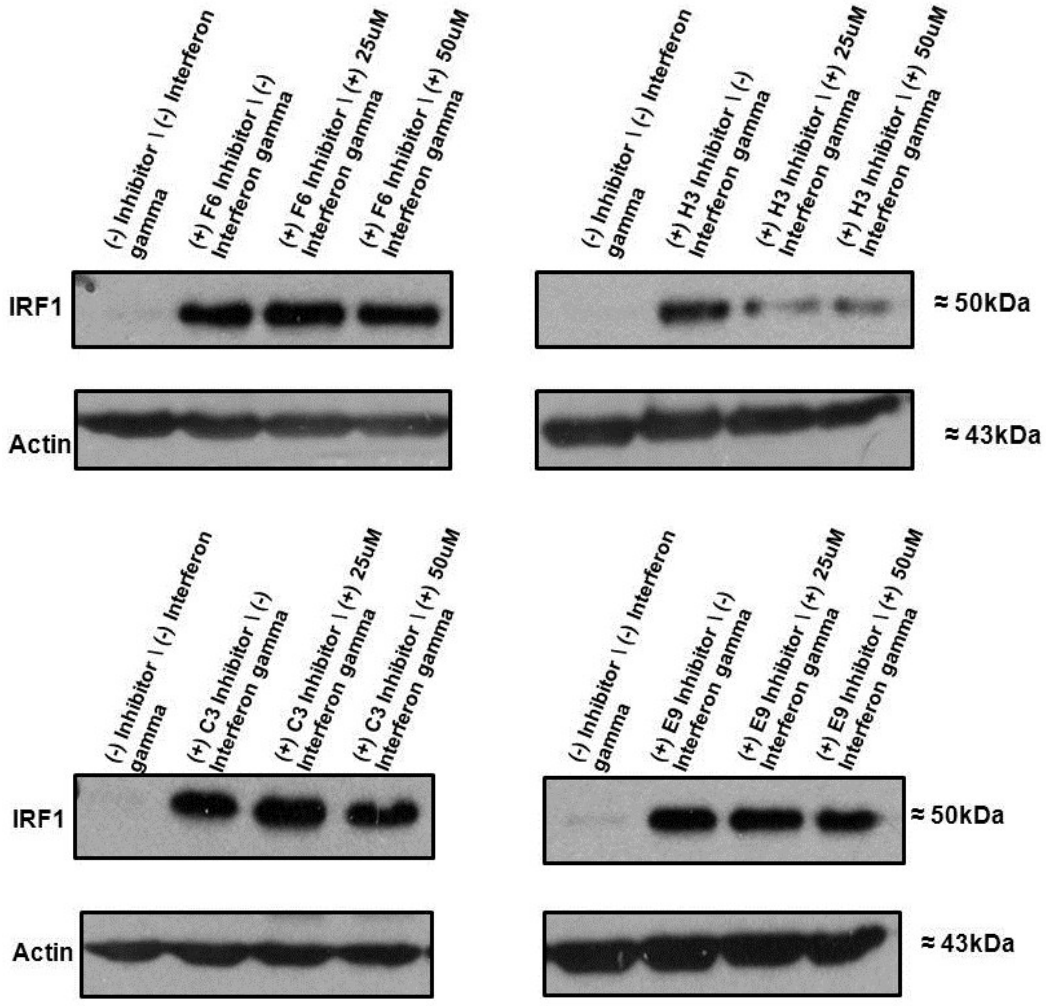
(B)

Figure 5.5: IRF-1 transcriptional activity in A375 FRT7-Luci-MIX. (A) Reporter cell line was treated with 5, 25, and 50μM of C3, F6, H3, or D11 followed by measuring IRF-1 transcriptional activity using luciferase. (B) IRF-1 protein levels were detected using immunoblotting after kinase inhibitor treatment.

To test whether these kinase inhibitors have an effect on IRF-1 transcriptional activity after induction using INF-γ, A375 FRT7-Luci-MIX cells were seeded onto 6-well tissue culture plates 18hr pre treatment. The following day cells were treated with 2units/ml INF-γ and either 25μM or 50μM of C3, E9, F6, or H3 for 24hr. Following treatment, protein lysate was subjected to immunoblotting and luciferase activity measurement (Figure 5.6). Results shows that C3 overcame the induction of IRF-1 by interferon-γ and thus confirmed the reproducibility, even at a low dosage (25μM) where F6 is further stimulating IRF-1 transcriptional activity. There was no change in IRF-1 transcriptional activity after E9 kinase inhibitor treatment, and the H3 effect is only seen at high dosage (50μM). IRF-1 protein level correlates with interferon-γ activity.


Luciferase activity
C3
E9
F6
H3
Kinase Inhibitor
(-) Inhibitor (-) Interferon
(-) Inhibitor (+) Interferon
(+25uM) Inhibitor (+) Interferon
(+50uM) Inhibitor (+) Interferon

(A)

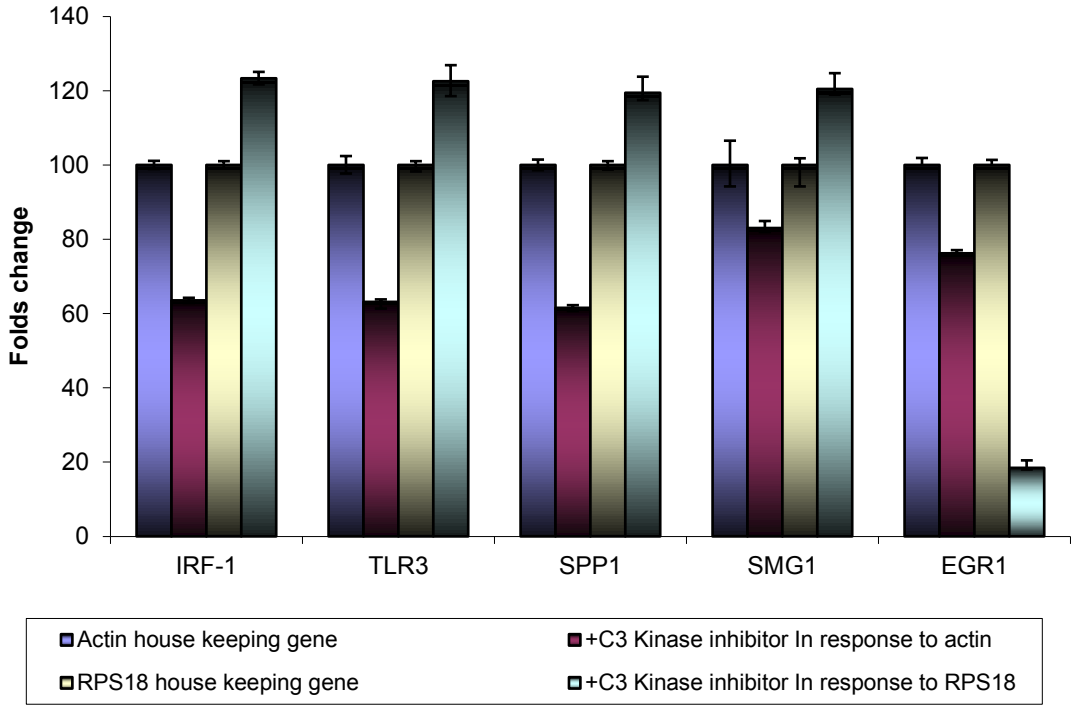


(B)

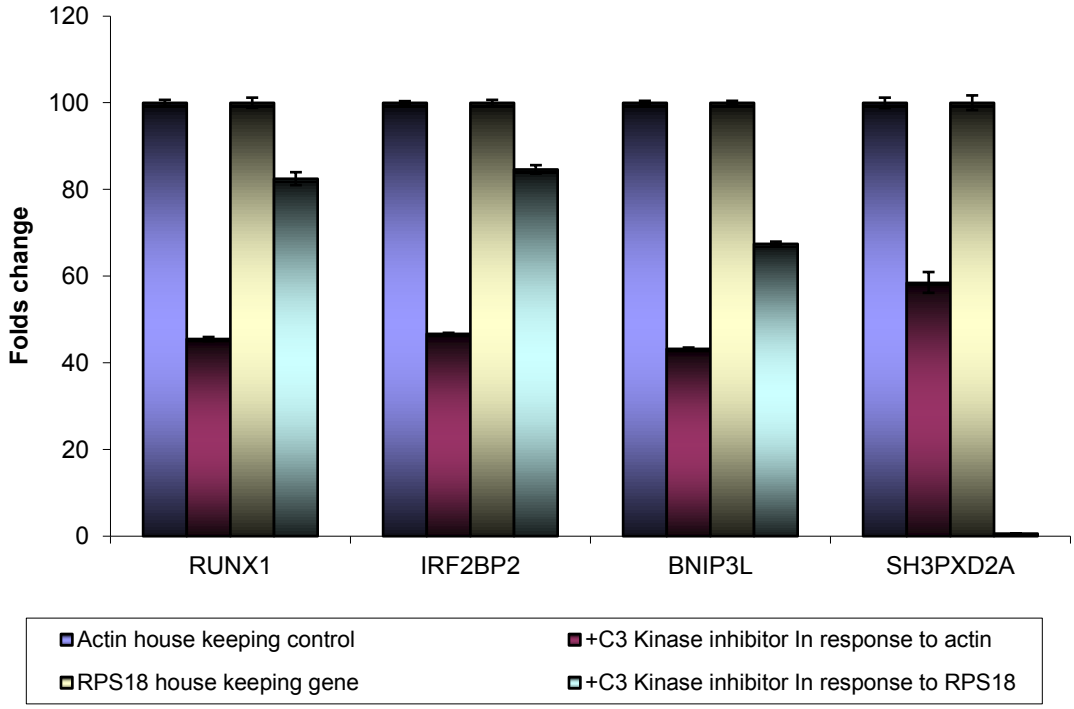
Figure 5.6: IRF-1 transcriptional activity in A375 FRT7-Luci-MIX. (A) Cell line was treated with 2units/ml of INF-γ and 25μM or 50μM of C3, E9, F6 or H3, followed by measuring IRF-1 transcriptional activity using luciferase. (B) IRF-1 protein levels were detected using immunoblotting after kinase inhibitor treatment.

5.3.3 The effect of VEGFR tyrosine kinase inhibitors on IRF-1 downstream targets

To test the effect VEGFR (C3) inhibitor on IRF-1 downstream target genes, A375 FRT7 conIRF-1 was seeded onto 6-well tissue culture plates 18hr prior to treatment. The following day cells were treated with 25µM or 50µM of C3 for 12hr. After treatment, mRNA was extracted from A375 FRT7 conIRF-1 and used for qRT-PCR (Figure 5.7) as explained before.



(A)



(B)

Figure 5.7: Quantitative real-time PCR analysis of 7 IRF-1 target genes. (A) First round of screening where 3 target genes SPP1, SMG1, and EGR1 show clear 20%-40% down regulation in response to actin house keeping gene once A375 FRT7 conIRF-1 treated by VEGFR kinase inhibitor. (B) Second round of screening where 4 target genes RUNX1, IRF2BP2, BNIP3L, and SH3PXD2A show clear 45%-90% down regulation in response to actin or RPS18 house keeping gene once A375 FRT7 conIRF-1 treated by VEGFR kinase inhibitor. IRF-1 and TLR3 positive control show clear down regulation in response to actin but not RPS18.

5.4 Discussion

We have successfully created a stable reporter cell line that can be used for high throughput screening for IRF-1 modifiers. We attempted to create a reporter cell line using Flp-In™ System as described previously, however, we found that luciferase activity was un-detectable, suggesting that the Flp-In™ System is only practicable for protein coding sequences and not regulatory sequences. Therefore we attempted a different method using traditional cloning. Both clones (A375 FRT7-Luci-MIX and A375 FRT7-Luci-clone 4) showed luciferase activity when the reporter cell lines were treated with interferon-γ (Figure 5.3 and 5.4); such stimulation of IRF-1 is transduced *via* IFNAR2 and IFNGR1 receptors through the GAF/AAF complex (Figure 5.2). The IRF-1 protein level is over expressed, in correlation with interferon-γ dosage, when compared to non-treated cells (Figure 5.3.B and 5.4.B). In addition, TLR3 stimulation depends on similar IRF elements, which constitutively bound IRF-2 and recruited IRF-1 after stimulation (Heinz *et al*., 2003). Thus, the use of TLR3 regulatory elements can report the activity of IRF-1.

The luciferase cassette was integrated randomly in the genome of A375 FRT7, resulting in different clones with different luciferase activity; the mixed clone showed better luciferase activity when compared to clone 4 and thus the mixed clone was chosen. Five units/ml of interferon-γ is sufficient to induce IRF-1, whereas more than this causes receptor saturation with undetectable differences (Figure 5.3.A and 5.4.A). Treatment time varied between 4–24hr, where 2units/ml of interferon-γ for 16hr was the optimum (data not shown).

The first round of screening revealed that 40 different kinase inhibitors caused a decrease in IRF-1 transcriptional activity whereas 8 kinase inhibitors caused an increase in its activity. However, the remaining 32 inhibitors showed no significant change in IRF-1 transcriptional activity (data not shown). Among those that showed either a stimulation or repression, we picked six kinase inhibitors that showed an interesting pattern after the first round of screening (table 5.1). We also noticed that, within the picked group, more than one kinase inhibitor hit the same target (H3 and E4 target EGFR) where (C3 and E9 target VEGFR). EGFR, VEGFR, and FLT3 are all members of a large receptor family named receptor tyrosine kinases (RTKs), suggesting a role of RTK in IRF-1 signalling.

The RTK family comprises cell-surface receptors that regulate proliferation, differentiation, cell survival, metabolism, cell migration, and the cell cycle (Blume-Jensen and Hunter, 2001). Humans have 58 known RTKs, falling into 20 different families, including FLT3, EGFR and VEGFR. RTKs have a wide role in human diseases, where RTK pathway disturbances by genetic mutation have been linked to cancer, diabetes, inflammation, severe bone disorders, arteriosclerosis, and angiogenesis (Lemmon and Schlessinger, 2010). Angiogenesis is an important process in tumour development (Risau, 1997) as the development of new blood vessels from pre-existing vasculature is essential for supplying oxygen and nutrients for the growing tumour (Yancopoulos *et al*., 2000). VEGF is a key player in angiogenesis as it stimulates proliferation and migration in endothelial cells (Ferrara *et al*., 2002).

The mechanism of action of kinase inhibitors used in this study has been revealed; kinase inhibitors used here do not influence the IRF-1 protein levels (Figure 5.5) thus, all findings were based on reduction in IRF-1 transcriptional activity and not reduction in IRF-1 protein levels. As a result, these kinase inhibitors influence IRF-1 transcriptional activity. In addition, we also revealed that these kinase inhibitors could reduce (C3) or stimulate (F6) interferon-γ stimulated IRF-1 transcriptional activity (Figure 5.6). Thus, we propose that these kinase inhibitors target proteins that cooperate and/or play a role in IRF-1 transcriptional activity. To further confirm this hypothesis, we investigated the effect of these kinase inhibitors on other IRF-1 downstream target genes identified in chapter four. We chose the C3 kinase inhibitor as it showed reproducible results throughout all screening rounds, and shows its reduction effect at very low concentrations (0.4μM).

We confirmed the reduction in the mRNA levels of IRF-1 downstream targets following C3 treatment (Figure 5.7) and thus confirmed C3 as a modifier that disturbs IRF-1 transcriptional activity, leading to the reduction in the transcription of IRF-1 downstream target genes. The kinase inhibitor C3, at the concentration used, targets VEGFR and PDGFR tyrosine kinases, where one or both seem to play a role in regulating IRF-1 transcriptional activity in melanocytes.

C3 inhibitor targets both PDGFR and VEGFR tyrosine kinases, however it was important to dissect the pathway to identify the exact IRF-1 modifiers, therefore new, potent and selective inhibitors specifically targeting PDGFR, VEGFR, EGFR or FLT3 were obtained (Merck) and a further round of screening performed (data not shown). Results of this revealed that IRF-1 transcriptional activity is reduced when treated with PDGFR and EGFR inhibitor but not with VEGFR inhibitor, whereas IRF-1 transcriptional activity was increased when treated with FLT3 inhibitor. However, immunoblotting revealed that IRF-1 protein levels were reduced as a result treatment with PDGFR, VEGFR, and EGFR kinase inhibitors. Thus, the study using the Merck inhibitors was discontinued, as they influence IRF-1 protein levels and not its transcriptional activity.

There is no evidence in the literature for a link between IRF-1 and RTK; however, it has been reported that IRF-1 as a transcription factor induces EGFR promoter activity up to 200-fold, compared to a 3–10-fold induction by other regulators (Rubinstein *et al*., 1998). Thus, IRF-1 has been identified as a major regulator of EGFR gene expression. In addition, IRF-1 has been found to inhibit endothelial cell angiogenesis, inhibit tube formation, and reduce the expression of angiogenesis proteins following VEGF stimulation (Lee *et al*., 2008). As a result, IRF-1 has proven to have an anti-angiogenic role, which may have therapeutic value for cancer and angiogenesis-associated disorders.

Within the RTK family, EGFR, PDGFR, and VEGFR share a common regulatory mechanism involving receptor dimerization and activation of tyrosine kinase, as well as creation of docking sites for signal transduction (Olsson *et al*., 2006). RTK signalling is thought to be a complicated and interlinked network, as some RTKs, such as EGFR, have multiple (5–12) auto-phosphorylation sites that could each recruit different SH2 domain-containing proteins (Lemmon and Schlessinger, 2010); in addition, a given adaptor or a scaffold protein can interact with multiple signalling molecules (Schlessinger, 2000) suggesting that RTK signalling is highly interconnected, forming a complex and dynamic signalling network.

Stimulation of EGFR can initiate the transcription of downstream target genes by the formation of the STAT1 and STAT3 transcription complex (Lemmon and Schlessinger, 2010); however, it has been reported by Anderson *et al* (2008) that stimulation of EGFR can induce IRF-1 expression in human cancer cells in which signalling cascades of other members of the TRK family, such as VEGFR, could be also present.

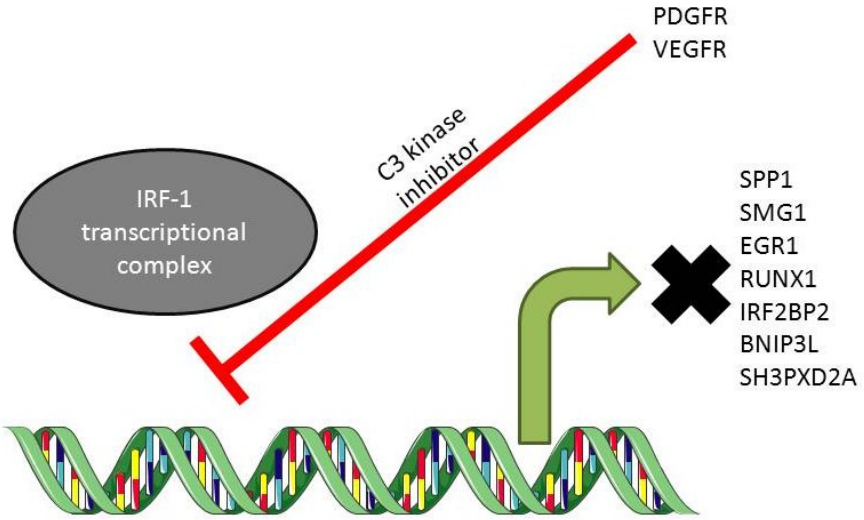


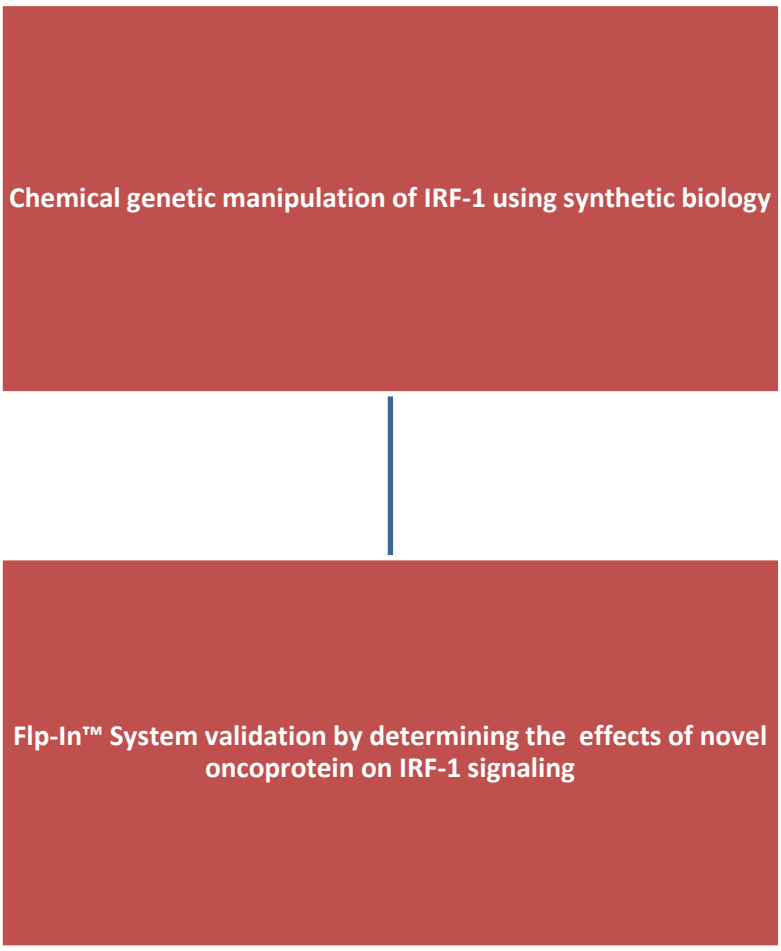
Figure 5.8: Schematic illustration of IRF-1 modifier in melanocytes. C3 has been identified as an IRF-1 modifier that regulates IRF-1 transcriptional activity by inhibiting PDGFR and/or VEGFR. As a result, IRF-1 downstream targets are reduced or inhibited.

We have proposed a model (Figure 5.8) where C3 as an IRF-1 modifier can regulate IRF-1 transcriptional activity. This regulation is mediated by inhibiting PDGFR and/or VEGFR tyrosine kinase. As a result, IRF-1 downstream targets including TLR3, SPP1, SMG1, RUNX1, IRF2BP2, and BNIP3L are reduced at their mRNA levels, whereas SH3PXD2A and EGR1 are inhibited at their mRNA levels. It is still unknown exactly how PDGFR and/or VEGFR inhibition by C3 influences IRF-1 transcriptional activity and, further testing will be required to reveal the regulatory mechanism. Meanwhile, the A375 FRT-Luci-MIX has been proven to be an excellent synthetic reporter tool for high throughput screening for identifying new IRF-1 modifiers.

Chapter 6: Synthetic cassette to study effects of a novel oncoprotein on IRF-1 signalling: Identification of AGR-2 as a novel inhibitor of IRF-1 protein stability

6.1 Chapter abstract

To further validate the synthetic Flp-In™ cassette, we decided to test the system using a novel oncoprotein model. We have developed a stable cell line that overexpresses an oncoprotein named anterior gradient-2 (AGR-2). This novel AGR-2 model was used to test the effect of such a model on a tumour suppressor (IRF-1) activity. Results show that both poly (I:C) and AGR-2 suppress p53 and IRF-1 whereas interferon-γ overcame the suppression, as interferon-γ is p53-independent. We also identified AGR-2 as a cellular survival element. In conclusion, the Flp-In™ cassette has been validated and it can be used as a tool for cancer studies.



6.2 Chapter Introduction

Intracellular organelles play an important role in tumorigenesis and cancer progression. One important intracellular organelle in this regard is endoplasmic reticulum (ER) (Moenner *et al*., 2007). ER is an organelle in eukaryotic cells that forms an interacting network of tubules and vesicles where protein and lipid synthesis, regulation of calcium, carbohydrate metabolism, and drug detoxification take place (Chevet *et al*., 2001). To achieve and maintain such functions, ER must coordinate the actions of protein translocation, protein folding, protein export, protein degradation and ER stress signalling (Chevet *et al*., manuscript in preparation). Some of the proteins controlling ER functions have been shown to acquire a role in cancer development (Moenner *et al*., 2007). The same observation has been noted for components of oxidative protein folding which includes the protein disulphide isomerase (PDI) family (Chevet *et al*., manuscript in preparation and Benham *et al*., 2011). The human PDI protein family has 20 members and is characterized by the presence of thioredoxin motifs CXXC. AGR-2 belongs to the PDI family. In this chapter, a stable cell line overexpressing human AGR-2 is developed in which the effect of its overexpression on the IRF-1 tumour suppressor is studied.

6.2.1 The AGR gene family

The anterior gradient (AGR) gene family is composed of three members named AGR-1, AGR-2, and AGR-3, where AGR-2 is the most studied and understood. The AGR family was first identified in *Xenopus laevis* and named *XAG-1*, *XAG-2*, and *XAG-3*. *XAG-1* and *XGA-2* have a specific expression pattern in the cement gland, and XAG-2 specifies the fate of the dorso-anterior ectoderm and induces the development of the forebrain (Chevet *et al*., manuscript in preparation). In addition, XAG-2 acts as signalling molecule inducing cement gland differentiation. It has been reported that, during early development, *XAG-1* and *XAG-2* are expressed in a gradient (hence the name) within ectoderm-derived organs such as the cement gland, a mucus secreting gland located at the anterior of the frog head (Aberger *et al*., 1998).

Anterior gradient proteins have subsequently been identified in zebrafish, pufferfish, Atlantic salmon, human and mouse (Shih *et al*., 2007). In mouse embryos, mAGR-2 (XAG-2 homologue) is highly expressed at the mRNA level in gut: mainly stomach, intestines, and colon (Komiya *et al*., 1999). Using proteomic analysis of multiple human breast tumour-derived cell lines, AGR-3 was first identified in humans as a unique protein named BCMP11, later named AGR-3. AGR-3 has no known function but its expression correlates with oestrogen receptor in human breast cancer (Adam *et al*., 2002). AGR-3 and AGR-2 show 71% sequence identity and both are located in the same genomic segment (Chevet *et al*., manuscript in preparation). However, AGR-2 has the more critical role in carcinogenesis, as AGR-3 has not been identified in many human cancers (Chevet *et al*., manuscript in preparation). The third and final group member is AGR-1, identified using its similarity to classic thioredoxin fold proteins containing the CXXC motifs (Alanen *et al*., 2003). AGR-1 functions in the formation of disulfides with client proteins in the ER (Jessop *et al*., 2009), it has not, however, been identified in human cancer and thus its role in tumorigenesis is still unknown (Chevet *et al*., manuscript in preparation).

6.2.2 The AGR2 member of the AGR family

In normal cells, AGR-2 appears to be expressed in mucus secreting cells located in organs derived mainly from endoderm; these cells ensure tissue homeostasis and integrity. In addition, AGR-2 has been reported to be stimulated by oestrogen and found to be up-regulated in response to oestradiol in epithelium and stroma of normal human breast tissue (Thompson *et al*., 1997 and Wilson *et al*., 2006). In addition to AGR-2 stimulation by oestrogen, other groups have reported direct binding of transcription factors including forkhead fox (FOXA1 and FOXA2), hepatic nuclear factor 1 (HNF1), ZNF217, and NF-κB, and SOX9 to the AGR-2 regulatory element. Most of these transcription factors have been implicated in maintaining goblet cell function (Zheng *et al*., 2005 and Krig *et al*., 2007).

In terms of ligand and receptors, AGR-2 has been reportedly induced following treatment with tunicamycin, an inhibitor of N-linked glycosylation and an inducer of ER stress (Zweitzig *et al*., 2007) through the activation of ATF6 and IRE1 arms of the unfolded protein response (UPR) (Higa *et al*., 2011).

In addition to tunicamycin, AGR-2 has been reportedly induced by androgen, where this induction is time and dosage dependent and requires the presence of androgen receptor (Zhang *et al*., 2005).

The biological role of AGR-2 is unknown; however, possible functions may be linked to AGR-2 as a result of its similarity with XAG-1 and XAG-2, proposing an involvement in the proliferation of mammalian tissue. In addition, Liu *et al* (2005) reported that AGR-2 could mediate metastasis in animal models and this proposal was validated when Dumartin *et al* (2011) showed that AGR-2 is a cell surface antigen, promoting tumour cell dissemination through the activation of cathepsins B and D.

6.2.3 AGR-2 and cancer

To investigate the role of AGR-2 in cancer, assays that involve overexpression and/or suppression of AGR-2 have been employed. Results showed that AGR-2 exhibits the basic features of a pro-oncogenic activity. AGR-2 can enhance cancer cell survival rather than inhibit cell growth (Hrstka *et al*., 2010). Subsequently, AGR-2 silencing was shown to inhibit proliferation, invasion and survival *in vitro* in pancreatic (Ramachandran *et al*., 2008) and breast cancer cell lines (Vanderlaag *et al*., 2010). Also, AGR-2 has been described by Wang *et al* (2008) as an oncogene, supporting a role in cellular transformation and adenocarcinoma growth.

Tamoxifen is an anti-oestrogen used to treat human breast cancers that are mainly estrogen receptor positive (ER+); however, acquired resistance to Tamoxifen in cancerous cells is a rising problem in breast cancer management. It has been suggested that some genes that might contribute to Tamoxifen resistance may act as agonists of Tamoxifen, thus minimizing its activity and functionality. From a pharmaceutical point of view, identifying oestrogen-responsive genes that are induced by Tamoxifen have been a key aim in resolving the puzzle of Tamoxifen resistance in breast cancer.

Reasons for suggesting that AGR-2 is one of the genes that mediate Tamoxifen resistance in breast cancer, include observations in cancer patients treated with the oestrogen suppressor "Letrazole". The AGR-2 gene was one of the main suppressed genes in biopsies from patients that respond well to the drug, suggesting that resistance to anti-oestrogen might be due to failure to suppress AGR-2.

In addition, AGR-2 was second in the list of genes induced by Tamoxifen in a proteomics screen. Further investigation using chromatin immunoprecipitation revealed that this induction is a result of direct activation of ERα, which is bound to the AGR-2 promoter in the presence of Tamoxifen. Luciferase assays using an AGR-2 regulatory element confirmed the induction of AGR-2 when cells were treated with Tamoxifen. Understanding the molecular basis behind Tamoxifen selectivity in inducing AGR-2, while acting as an anti-oestrogen for the other oestrogen-responsive genes, might identify novel drug targets.

6.2.4 AGR2 and p53 tumour suppressor activity

Previous work in our lab has studied the relation between AGR-2 and p53. It is known that wild-type p53 is maintained in 30–80% of Barrett's metaplasia; however, we were interested in whether or not p53 function was being attenuated *via* an alternative mechanism. Studies showed AGR-2 is up-regulated in Barrett's metaplasia compared to normal oesophageal tissue, suggesting a role in oesophageal cancer progression. We also found that AGR-2 could function similarly to a mutant oncogenic p53 allele after noticing a similar pattern in enhancing colony formation when compared to a p53 mutant (HIS175 allele). In addition, the p53 transcriptional level was reduced when co-transfected with AGR-2, suggesting that AGR-2 might have a kinase inhibitory role, especially on Ser15 and Ser392 phosphorylation.

To further investigate this model linking AGR-2 and another tumour suppressor (IRF-1), and to validate the Flp-In™ cassette, we decided to create a stable cell line that overexpresses wild-type AGR-2 using the Flp-In™ System. This model will help to validate the Flp-In™ cassette and to facilitate whether AGR-2 can also attenuate other tumour suppressors such as IRF-1.

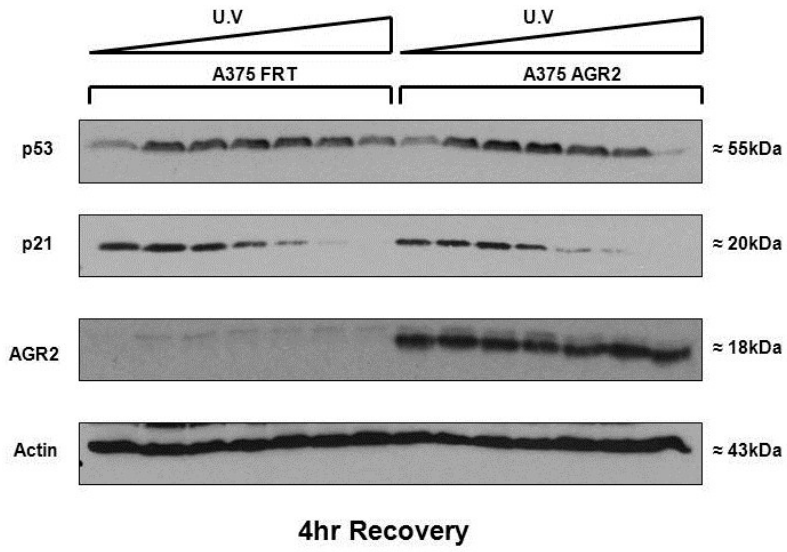
6.3 Methods and results

6.3.1 Creating a stable A375 Flp-In™ host cell line overexpressing AGR-2: AGR-2 attenuates p53 in human melanoma

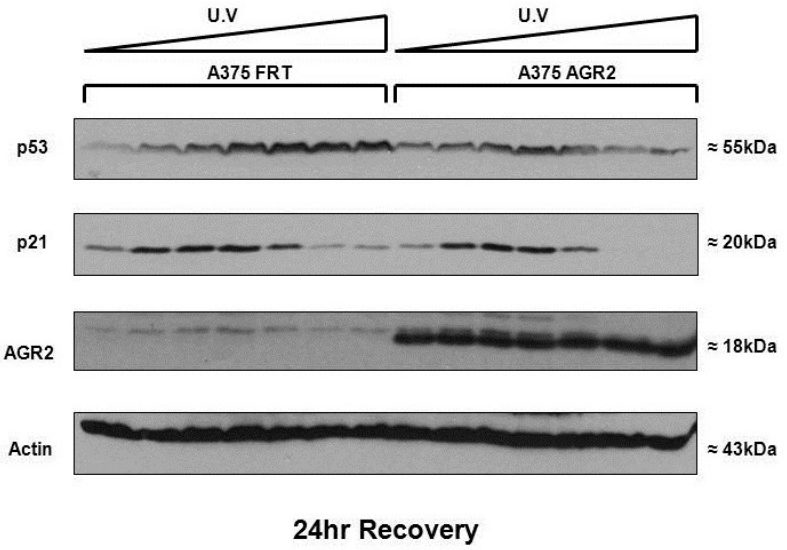
A stable cell line overexpressing human AGR-2 was created using the Flp-In™ System as described in chapter four. The pDONR221 carrying human AGR-2 cDNA was a kind gift from Dr. Euan Murray; cell lines were made by Asia Ansari, under my direct supervision, using the A375 FRT7 clone.

Once created, the stable cell line was tested for AGR-2 overexpression (data not shown). Once this was confirmed, both cell lines (A375 FRT and A375 AGR-2) were seeded onto 6cm tissue plates and exposed to an increasing dosage of UV radiation: 0, 1, 2, 5, 10, 20, and 40J/m^2. After treatment, cells were allowed to recover for either 4 or 24hr. Following recovery, cell lysate was subjected to immunoblotting (Figure 6.1).

Results showed that p53 protein level increased in response to UV treatment in both cell lines (A375 FRT and A375 AGR-2); however, p53 level is attenuated as a result of overexpressing AGR-2. This attenuation of p53 protein level is more noticeable after 24hr recovery compared to 4hr recovery (Figure 6.1.A and B-top panel); p21 protein levels correspond to p53 protein levels in both cell lines whereas a greater reduction in p21 level is noticeable in the cell line overexpressing AGR-2 after 24hr recovery (Figure 6.1.A and B-second top panel). Both AGR-2 and β-actin protein levels confirm overexpression of AGR-2 in A375 AGR-2 compared to the empty vector control when an equal amount of protein is loaded in each well.



(A)



(B)

Figure 6.1: The effect of UV on p53 in the presence of AGR-2. A375 FRT and A375 AGR-2 were seeded on 6cm culture plates and, 24hr later, cells were subjected to an increased dosage of UV (0, 1, 2, 5, 10, 20, and 40J/m^2) followed by either 4 or 24hr recovery. After harvesting, cells were lysed and subjected to immunoblotting. p53, p21, AGR-2, and β-Actin were detected by their respective antibodies.

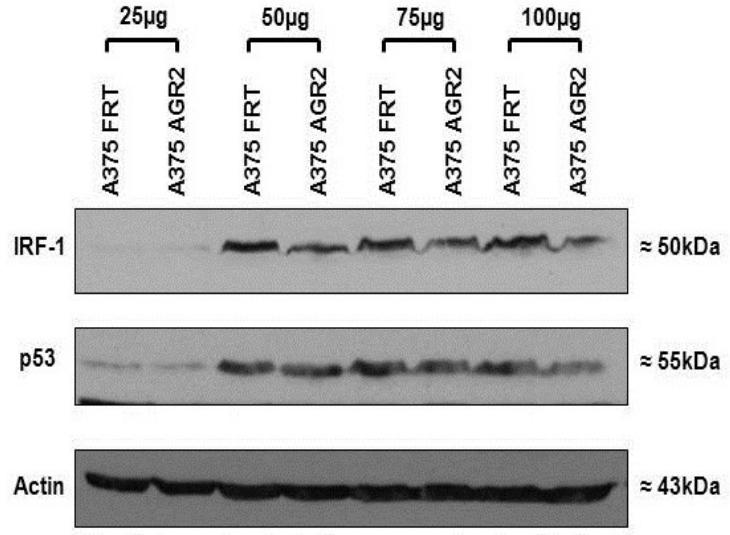
6.3.2 AGR-2 attenuates IRF-1 and p53 basal levels in human melanoma

To test the effect of AGR-2 overexpression on p53 and IRF-1 basal levels, A375 FRT and A375 AGR-2 were seeded onto 6cm tissue plates. The following day, cell lysate was subjected to immunoblotting (Figure 6.2A).

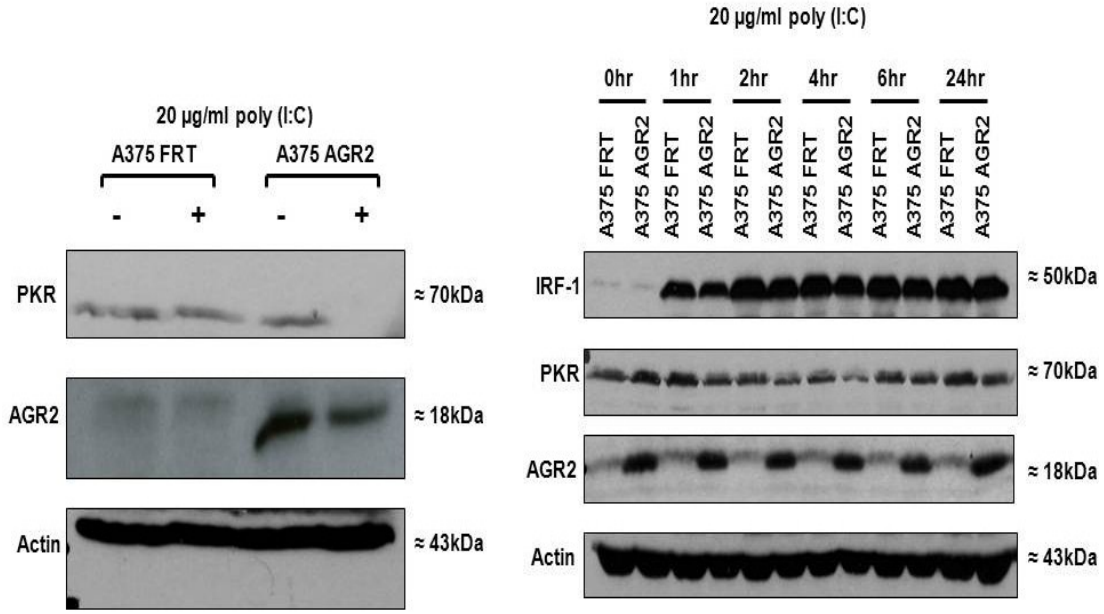
To test the effect of AGR-2 overexpression on IRF-1 induced by poly (I:C). A375 FRT and A375 AGR-2 were seeded onto 6cm tissue plates and treated with a fixed dosage of 20µg/ml of poly (I:C) for 4hr (Figure 6.2.B.Left) or a time course of 1, 2, 4, 6, and 24hr (Figure 6.2.B.Right). Following treatment, cell lysate was subjected to immunoblotting (Figure 6.2.B).

To test the effect of AGR-2 overexpression on interferon-γ induced IRF-1, A375 FRT and A375 AGR-2 were seeded onto 6cm tissue plates and treated with a fixed dosage of 2units/ml of interferon-γ for a time course of 1, 2, 4, 6, and 24hr (Figure 6.2.C). Following treatment, cell lysate was subjected to immunoblotting (Figure 6.2.C).

Results show that AGR-2 overexpression attenuates both IRF-1 and p53 basal levels and the effect is clearly shown when 50µg or more of total protein lysate is used (Figure 6.2.A). AGR-2 attenuates the induction of the IRF-1 pathway that is induced by 20µg/ml poly (I:C) at 4hr fixed time point (Figure 6.2.Right). This is also confirmed using a downstream target of IRF-1; the double-stranded RNA-activated protein kinase R (PKR) is attenuated as a result of AGR-2 overexpression (Figure 6.2.Left). Interferon-γ seems to overcome the AGR-2 overexpression signal as IRF-1 and PKR protein levels do not change in response to AGR-2 overexpression (Figure 6.2.C).

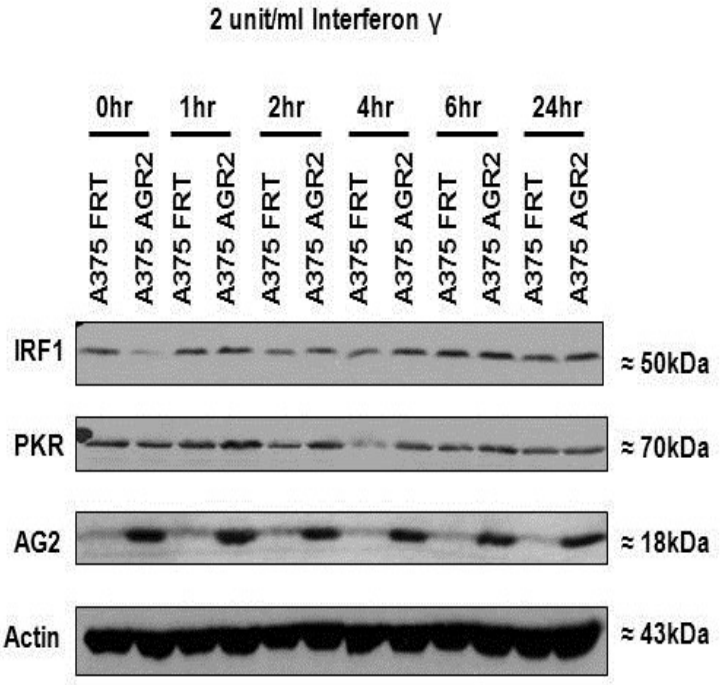


(A)



(Left) (Right)

(B)



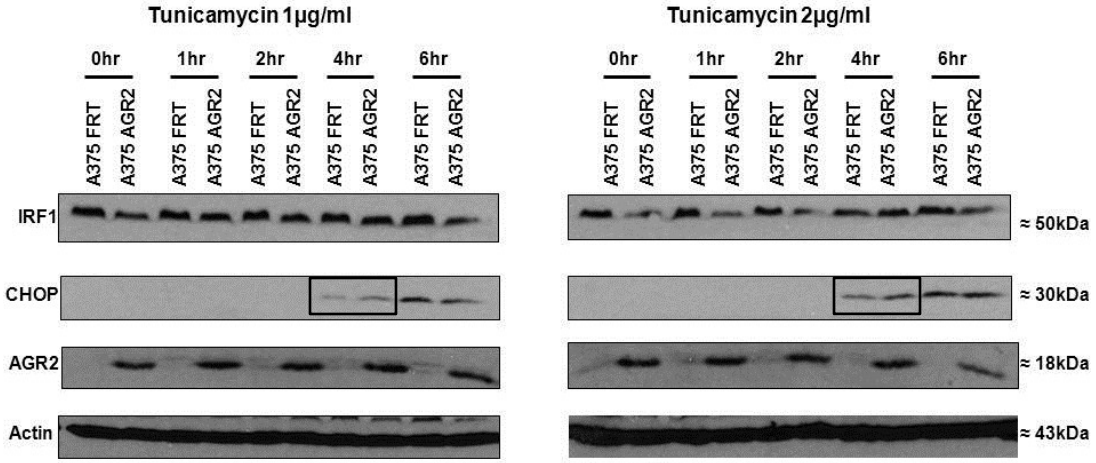
(C)

Figure 6.2: AGR-2 attenuates IRF-1 and p53 basal levels. (A) A375 FRT7 and A375 AGR-2 were seeded on 6cm culture plates; 24hr later, cells were subjected to immunoblotting: p53 and IRF-1 basal levels are reduced as a result of AGR-2 overexpression. (B) A375 FRT7 and A375 AGR-2 were seeded on 6cm culture plates, 24hr later, cells were subjected to poly (I:C) treatment, PKR protein level is reduced as a result of AGR-2 overexpression. (C) A375 FRT and A375 AGR-2 were seeded on 6cm culture plates; 24hr later, cells were subjected to interferon-γ. Interferon-γ seems to overcome the effect of AGR-2 signalling on IRF-1 pathway.

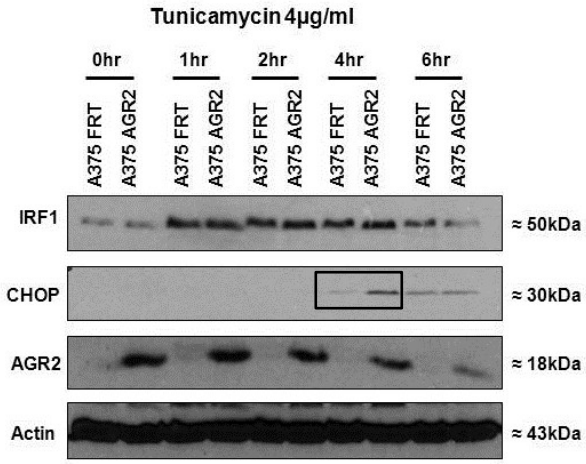
6.3.3 AGR-2: a cellular survival factor

To test the potential of AGR-2 as a survival factor, ER stress was initiated using tunicamycin. A375 FRT7 and A375 AGR-2 were seeded onto 6cm tissue plates and treated with 1, 2, or 4µg/ml tunicamycin for 0, 1, 2, 4, and 6hr. Following treatment, cell lysate was subjected to immunoblotting (Figure 6.3).

Results show that AGR-2 overexpression attenuates IRF-1 basal levels, where the effect is clearly shown during 2µg/ml tunicamycin treatment (Figure 6.3.A.Right). ER stress initiation following 4hr treatment is confirmed by C/EBP homologous protein (CHOP) overexpression (Figure 6.3). AGR-2 overexpression increases ER stress to sustain cellular survival, an effect confirmed by the increasing CHOP protein levels at 4hr time point (Figure 6.3.Box).



(A)



(B)

Figure 6.3: AGR-2: a cellular survival factor. A375 FRT7 and A375 AGR-2 were seeded on 6cm culture plates; 24hr later, cells were treated with 1, 2, or 4µg/ml tunicamycin for 1, 2, 4, and 6hr to initiate ER stress, followed by immunoblotting: IRF-1 basal level is reduced as a result of AGR-2 overexpression; ER stress is initiated by CHOP overexpression following 4hr of treatment. AGR-2 overexpression restores cellular survival, an effect noted by CHOP protein levels during AGR-2 overexpression (Box).

6.4 Discussion

Currently, we are investigating the proposed model of AGR-2 being a p53 kinase inhibitor, as discussed in the introduction; a stable cell line that overexpresses AGR-2 is a better tool than transient transfection. We have showed that the stable AGR-2 cell line responds as expected to UV irradiation. It is known that p53 accumulates in response to ionizing radiation (Kastan *et al*., 1991), where UV is also considered as a potent activator of p53 protein (Lu *et al*., 1993). Both cell lines, A375 FRT7 and A375 AGR-2, respond to UV in respect of p53 induction, and AGR-2 overexpression seems to attenuate this induction, especially at high UV (40 J/m^2) and with a longer recovery (24hr), compared to A375 FRT7 (Figure 6.1). The signalling mechanism in both UV and ionizing radiation involves some distinct steps; like ionizing radiation, UV also arrests cells in G1 phase (Li *et al*., 1996).

$p21^{WAF1}$ was identified as an inhibitor of cyclin dependent kinase and a gene whose transcription is enhanced by p53 (EL-Deiry *et al*., 1993). Here we report the reduction in p21 protein level as UV dosage increases; however, this p21 reduction is more noticeable in the cell line overexpressing AGR-2, where p53 is attenuated (Figure 6.1). Thus, AGR-2 overexpression attenuates p21 and p53 protein levels following UV treatment and after 24hr recovery. The Flp-In™ cassette was validated using p53-AGR-2 model and thus the cassette has proven to be a great tool for use in cancer biology.

Some studies showed that p21 decreases after UV irradiation of human tumour cells (Maki *et al*., 1997 and Allan *et al*., 1999) where other studies showed an increase in p21 protein after UV irradiation of fibroblasts in the absence of p53 (Loignon *et al*., 1997 and Haapajrvi *et al*., 1999). To reconcile this conflict it was proposed that p21 protein levels increase or decrease after UV irradiation depending on the cell type or the level of transformation (Fotedar *et al*., 2004).

Other studies showed that low UV (<40 J/m^2) leads to p21 degradation in diploid untransformed fibroblast (Bendjennat *et al*., 2003). Thus UV-induced degradation of p21 protein at low doses is independent of cell transformation and p53 although, p21 transcription increases after low dose UV irradiation of cells containing p53, where p21 protein is still degraded (Fotedar *et al*., 2004).

To test whether AGR-2 has an attenuating effect on other tumour suppressers, we tested the basal level of IRF-1 during AGR-2 overexpression (Figure 6.2.A). Results confirmed that IRF-1 basal protein level is reduced when AGR-2 is overexpressed, suggesting the ability of AGR-2 to act as a survival factor. This finding is similar to our observation with p53 and AGR-2, as studies in our lab suggested p53 transcriptional activity is reduced when co-transfected with AGR-2. This was found to be caused by AGR-2 acting as a kinase inhibitor, which blocks the phosphorylation of serine 15 and serine 392 modified by ataxia telangiectasia mutated (ATM) and CK2 respectively.

We have confirmed that this attenuation affects IRF-1 transcriptional activity, as PKR protein level was reduced when AGR-2 is overexpressed (Figure 6.2). PKR is known as a serine/threonine kinase with tumour suppressor activity, where its induction is caused by IRF-1 activation (Kirchhoff *et al*., 1995). Here we showed AGR-2 overexpression attenuates IRF-1 protein levels, resulting in reduction of its transcriptional activity.

We have not examined IRF-1 phosphorylation during AGR-2 overexpression, thus we are not sure whether this attenuation is p53 dependent or independent. Therefore, we decided to investigate the effect of AGR-2 overexpression on interferon-γ and poly (I:C)-induced IRF-1 (Figure 6.2.B and C). We have found the attenuation effect is only noticeable on poly (I:C) treatment but not on interferon-γ treatment. Thus, we propose that the IRF-1 attenuation effect is p53 dependent, as poly (I:C) is p53 dependent and interferon-γ is p53 independent.

Tanka *et al* (1996) first reported the cooperation between IRF-1 and p53 in response to DNA damage. It is known that their expression is independent of one another, however, both are required for p21 transcription, as the p21 promoter is activated, directly or indirectly, by both. Later, Dornan *et al* (2004) reported that IRF-1 and p53 synergize at the p21 promoter, and p21 transcription relies on the ability of IRF-1 to bind the coactivator p300 and to stimulate p53 dependent transcription.

A further hypothesis was based on the reports that showed that IRF-1 regulation during genotoxic stress involves the ATM signalling pathway (Pamment *et al*., 2002) or CKII signalling (Lin and Hiscott 1999). Here IRF-1 attenuation could be caused directly by AGR-2 acting as a kinase inhibitor of ATM and/or CKII kinetic activity (Figure 6.3). Interferon-γ has managed to overcome the AGR-2 signal, resulting in stabilization of IRF-1 transcriptional activity and proposing a potential therapeutic use by its ability to restore cellular tumour suppressor activity and overcome an oncogenetic signal. However, interferon-γ has a toxic effect on patients, thus reducing its potential in clinics (Sriskandan *et al*., 1986).

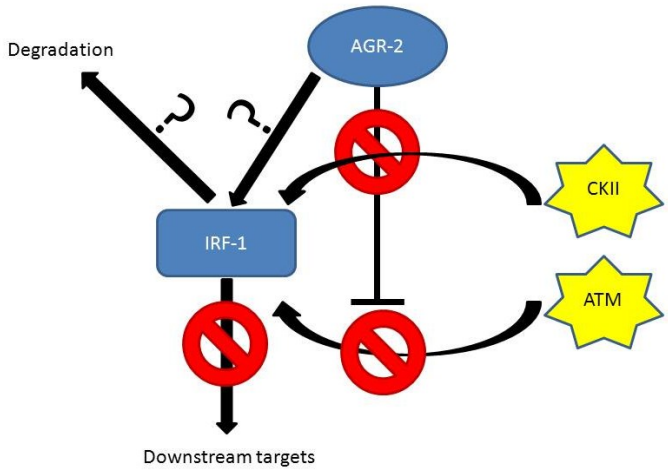


Figure 6.4: Proposed model for IRF-1 attenuation resulting from AGR-2 overexpression. AGR-2 can act as a kinase inhibitor resulting in blocking the phosphorylation of IRF-1 by CKII and/or ATM and leading to the reduction in IRF-1 transcriptional activity.

Protein folding in the ER could be impaired under various physiological and pathological conditions called ER stress (Kaufman, 1999). To overcome such ER stress, cells can initiate a response by a specific signalling pathway, termed the ER stress response pathway, which involves at least four responses (Ron, 2002). The fourth is apoptosis, which occurs when ER is severely impaired, and which protects the organism by eliminating damaged cells (Ferri and Kroemer, 2001; Oyadomari *et al*., 2002). One of these apoptotic pathways is the CHOP pathway leading to the activation of caspase-3, suggesting that ER stress signals are finally transmitted to the mitochondria (Oyadomari and Mori 2004). Thus CHOP overexpression is an indication of the ER response indicating an attempt at cellular survival before forcing the final choice of apoptosis.

Here we have reported the initiation of the ER stress response by CHOP overexpression following tunicamycin treatment (Figure 6.3). AGR-2 overexpression in A375 AGR-2 enhances CHOP overexpression as a result of tunicamycin treatment; however, this is only noticeable at the 4hr time point (Figure 6.3.Box). This observation proposed a role of AGR-2 overexpression is sustaining cellular survivors. In addition, an identical observation has been noted when AGR-2 was knocked-down in Hela cells using AGR-2 siRNA; the levels of CHOP were down-regulated when cells were treated with tunicamycin for 8hr (Higa *et al*., 2011).

A further study using different ER stress inducers, thapsigargin, which promotes ER stress by depletion of ER calcium stores, and dithiothreitol which disrupts disulphide bond formation, has showed that the proliferation rate and cellular survival rate increases once wild-type AGR-2 is overexpressed during thapsigargin treatment (E. Murry unpublished date). All these finding from our lab suggest an important role for AGR-2 in cellular survival during ER stress response.

Chapter 7: Final conclusions and future perspective

This study described the creation and the validation of synthetic tools that can aid and speed progress in scientific research and development. We have used IRF-1 to validate these developed tools showing their potential for research use and drug development. First, we have outlined the steps used in designing a novel targeting cassette that can be used to create *cre*-conditioned mouse models using recombineering technology. We have successfully developed a pLSL-TOPO-BAC vector, a universal BAC targeting vector, that can be used for any gene of interest where homologous arms can easily be slotted in, followed by the creation of desired mutations. Once created, *cre*-conditioned mouse models are easily developed.

I have also discussed different methods used for desired mutation creation; however, I recommend two untested new methods named ZFNs or CompoZr® (zinc finger nuclease technology) and TALENs™ (transcription activator-like effector nucleases) to create the required mutation following STOP cassette recombineering into the desired BAC. ZFNs are a class of engineered DNA-binding proteins that have the ability to recognize and bind onto a specific DNA location facilitating target editing by creating double-strand breaks in DNA followed by sequence alteration at the desired site using the cellular natural DNA-repair process, namely homologous recombination and non-homologous end joining. Sigma-Aldrich provides a custom-made ZFN service for genomic engineering in any species of cell line. We recommend the use of such a service to obtain custom-made ZFN for desired genomic alterations on BACs following the STOP cassette integration. Once a BAC subtle mutation is created, the whole BAC fragment carrying the STOP cassette, desired mutation and homologous fragments can be slotted into a stem cell targeting vector, such as pL611 for embryonic stem cell use, prior to selection and blastocyst injection.

Similar to ZFNs, TALENs is a recent development in the field of gene-editing nucleases, where a DNA-binding motif has been identified in proteins termed transcription activator-like effectors (TALEs), which nucleases can be attached to (Baker 2011). These TALENs have been proven to be easier to design and capable of performing the same function of zinc finger proteins in ZFNs. TALEs have very similar structure, except for the one section in the middle. This section is highly repetitive, made up of sequences that are each about three dozen amino acids long (Baker 2011). Within each TALE, the repeat sequences differ from each other by two residues termed repetitive variable di-residues (RVD) (Baker 2011). Further, it has been noticed that the number of repeats in some TALEs was approximately equal to the number of nucleotides to which it binds (Baker 2011) leading to the hypothesis that each variable di-residue in a TALE repeat bound to a single nucleotide, which was later proven to be the TALE code (Baker 2011). The implication of TALE proteins was striking, as it means that proteins can easily be designed to bind any DNA sequence.

TALENs are easier to design than ZFNs, and other reports showed that TALENs are more specific and less toxic than ZFNs; however, this does not mean that TALENs are automatically the technology of choice. ZFNs have been used for more than a decade, but only recently nonspecific cutting across the genome has been reported. There is not enough evidence to declare that TALENs are a better technology than ZFNs as most research report the successful use of TALENs without reporting how frequently a TALEN fails to work. At the end, we seek a platform with the least off-target effects and the highest success rate.

Some companies such as Cellectis offer a TALENs™ custom service; however, the cost for customized TALENs or ZFNs exceeds the budget of a standard lab. Designing our own ZFNs or TALENs is not impossible as many public domains offer databases for ZF and TALEs DNA-binding sequences. However, labour, knowledge of this field, and time availability are all requirements for a successful TALEN or ZFN design.

The second part of this study showed the development of the Flp-In System™ from Invitrogen to ease the generation of isogenic stable mammalian expression cell lines. We have validated the system by creating two isogenic stable cell lines, one overexpresses wild-type IRF-1 and the second overexpresses a mutant that abolishes IRF-1 DNA-binding ability. Using microarray studies and quantitative real-time PCR, we have used the system to identify a new role of IRF-1 in cancer biology; we have successfully identified *SH3PXD2A* and *SPP1* as new IRF-1 target genes confirming a new biological role for IRF-1 in cellular adhesion, invasion, and tumour growth progression. In addition to *SPP1* and *SH3PXD2A*, we have identified new IRF-1 target genes with known biological roles of IRF-1 in autophagy, cell linage differentiation, apoptosis, tumour growth inhibition, DNA repair, signal transduction, and telomerase maintenance (Figure 4.11). Further *in-silico* analysis of the regulatory element of these identified genes revealed that it is highly likely that an IRF-1 binding domain, such as ISRE, is contained in the promoter region of those genes identified using microarray and qRT-PCR.

To further validate these results, we suggest the use of luciferase reporter technology where the regulatory element of these identified genes can be cloned downstream of a firefly luciferase gene on pGL3 backbone. Once cloned, a dual luciferase assay can report the activation of the regulatory element during IRF-1 overexpression and/or IRF-1 stimulation by interferon-γ or poly (I:C). This assay should confirm the direct activation of these genes by IRF-1 transcription factor to further validate our hypothesis.

To confirm the newly discovered role of IRF-1 in cellular adhesion and metastasis we suggest the use of commercially available cell invasion assays designed for cellular migration, invasion and wound healing. Using this assay, A375 conIRF-1 can be tested for its ability to enhance metastasis and invasion on comparison to it the control, A375 W11R, that abolishes DNA-binding ability and thus reduces IRF-1 blockage to SPP1 and/or SH3PXD2A.

The third tool we have developed in this study is the IRF-1 reporter cell line that can be used for high throughput screening. We have used this reporter cell line to screen a library of kinase inhibitors in order to identify new IRF-1 modifiers. Following many screening and validating rounds, we have identified a new IRF-1 modifier that regulates IRF-1 transcriptional activity by inhibiting platelet-derived growth factor receptor (PDGFR) and/or vascular endothelial growth factor receptor (VEGFR) tyrosine kinase. Phosphorylation of IRF-1 using these two tyrosine kinases has not been reported previously. However, we suggest further investigation by determining any potential of IRF-1 phosphorylation by these two tyrosine kinases using kinase activity assays, enzyme-linked immunosorbent assay (ELISA), in-gel kinase assays and *in vitro* protein kinase assay. Linking IRF-1 phosphorylation by these tyrosine kinases will prove the validity of such tools in the field of life science.

Last but not least, we have used the Flp-In System™ to generate a stable cell line that overexpresses an oncogene termed AGR-2. We validated the AGR-2 p53 model where previous work in this lab showed that AGR-2 can act as a kinase inhibitor preventing p53 phosphorylation by ATM and CKII and, thus, reducing p53 activity. We used the system to identify whether AGR-2 can attenuate the activity of another tumour suppressor, and we found that AGR-2 can attenuate IRF-1 basal protein levels as well as levels of poly (I:C) induced IRF-1 in human melanocytes. We proposed that this attenuation is p53 dependent, as other stimuli such as interferon-γ (p53 independent) can overcome the AGR-2 effect. In addition, AGR-2 has been identified as a cellular survival factor, where its overexpression aids cells to increase the production of proteins that play a role in unfolded protein responses.

In conclusion, this study described the creation and the validation of synthetic tools: synthetic cassette for *cre*-conditioned mouse creation, the Flp-In System™ for isogenic stable cell line creation, and IRF-1 reporter cell line for high throughput screening. All the synthetic tools were validated and used to investigate IRF-1, a transcription factor that plays a role in cancer and immune system.

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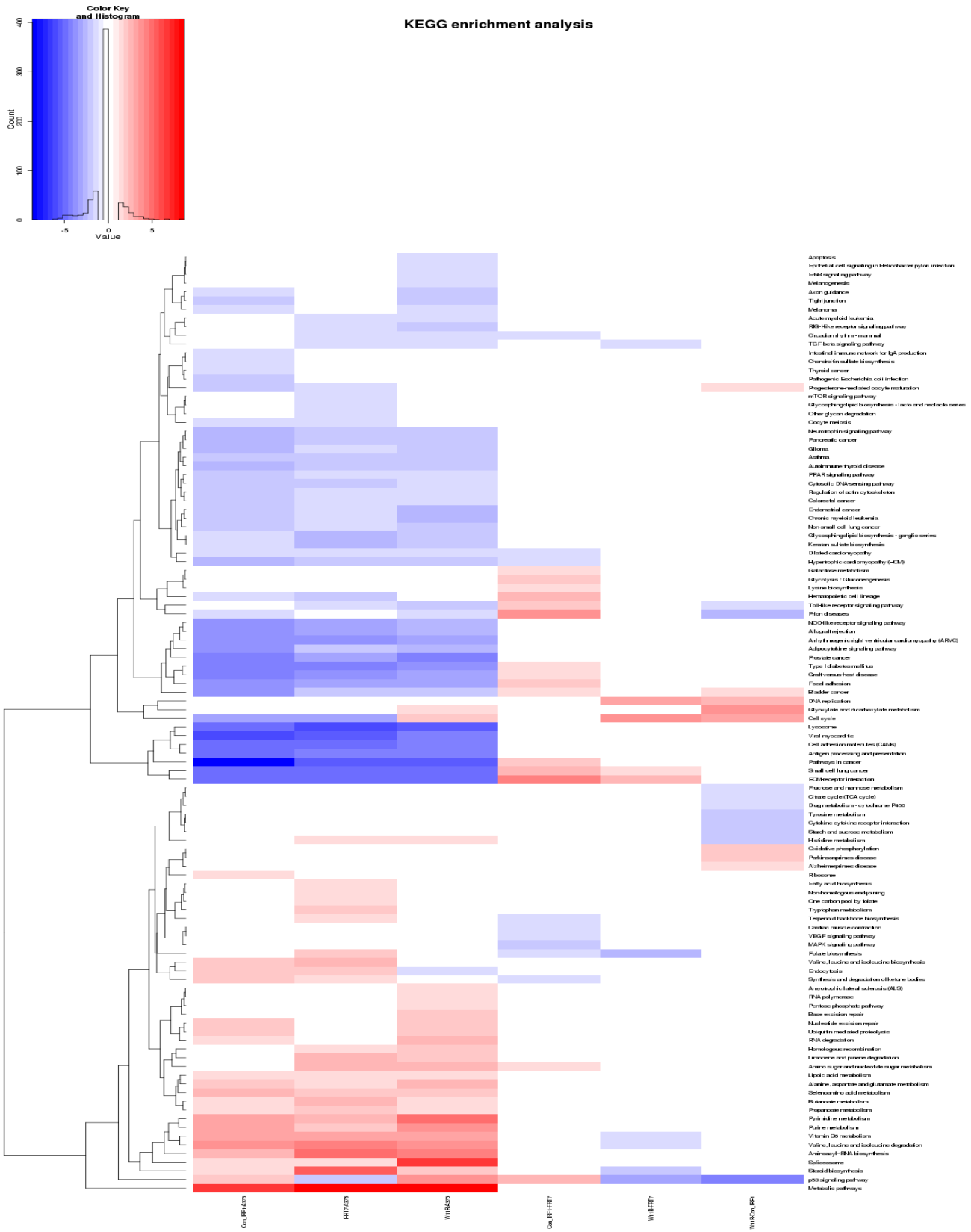
Appendix


Color Key and Histogram
Count
Value
KEGG enrichment analysis
Apoptosis
Epithelial cell signaling in Helicobacter pylori infection
ErbB signaling pathway
Melanogenesis
Axon guidance
Tight junction
Melanoma
Acute myeloid leukemia
RIG-I-like receptor signaling pathway
Circadian rhythm - mammal
TGF-beta signaling pathway
Intestinal immune network for IgA production
Chondroitin sulfate biosynthesis
Thyroid cancer
Pathogenic Escherichia coli infection
Progesterone-mediated oocyte maturation
mTOR signaling pathway
Glycosphingolipid biosynthesis - lacto and neolacto series
Other glycan degradation
Oocyte meiosis
Neurotrophin signaling pathway
Pancreatic cancer
Glioma
Asthma
Autoimmune thyroid disease
PPAR signaling pathway
Cytosolic DNA-sensing pathway
Regulation of actin cytoskeleton
Colorectal cancer
Endometrial cancer
Chronic myeloid leukemia
Non-small cell lung cancer
Glycosphingolipid biosynthesis - ganglio series
Keratan sulfate biosynthesis
Dilated cardiomyopathy
Hypertrophic cardiomyopathy (HCM)
Galactose metabolism
Glycolysis / Gluconeogenesis
Lysine biosynthesis
Hematopoietic cell lineage
Toll-like receptor signaling pathway
Prion diseases
NOD-like receptor signaling pathway
Allograft rejection
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Adipocytokine signaling pathway
Prostate cancer
Type I diabetes mellitus
Graft-versus-host disease
Focal adhesion
Bladder cancer
DNA replication
Glyoxylate and dicarboxylate metabolism
Cell cycle
Lysosome
Viral myocarditis
Cell adhesion molecules (CAMs)
Antigen processing and presentation
Pathways in cancer
Small cell lung cancer
ECM-receptor interaction
Fructose and mannose metabolism
Citrate cycle (TCA cycle)
Drug metabolism - cytochrome P450
Tyrosine metabolism
Cytokine-cytokine receptor interaction
Starch and sucrose metabolism
Histidine metabolism
Oxidative phosphorylation
Parkinson's disease
Alzheimer's disease
Ribosome
Fatty acid biosynthesis
Non-homologous end-joining
One carbon pool by folate
Tryptophan metabolism
Terpenoid backbone biosynthesis
Cardiac muscle contraction
VEGF signaling pathway
MAPK signaling pathway
Folate biosynthesis
Valine, leucine and isoleucine biosynthesis
Endocytosis
Synthesis and degradation of ketone bodies
Amyotrophic lateral sclerosis (ALS)
RNA polymerase
Pentose phosphate pathway
Base excision repair
Nucleotide excision repair
Ubiquitin mediated proteolysis
RNA degradation
Homologous recombination
Limonene and pinene degradation
Amino sugar and nucleotide sugar metabolism
Lipoic acid metabolism
Alanine, aspartate and glutamate metabolism
Selenoamino acid metabolism
Butanoate metabolism
Propanoate metabolism
Pyrimidine metabolism
Purine metabolism
Vitamin B6 metabolism
Valine, leucine and isoleucine degradation
Aminoacyl-tRNA biosynthesis
Spliceosome
Steroid biosynthesis
p53 signaling pathway
Metabolic pathways
Con_BF1-A375
FRT-A375
W11R-A375
Con_BF1-FRT7
W11R-FRT7
W11RCon_BF1